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LXIII. A MODIFICATION OF THE BARCROFT AND WINTERSTEIN MICRORESPIROMETERS.

By NEIL KENSINGTON ADAM[1].

From the Biochemical Laboratory, Cambridge.

(*Received September 31st, 1920.*)

The apparatus described in the present paper was designed for taking observations on the rate of absorption of oxygen by an isolated frog's muscle in any desired atmosphere, provision being made for simultaneous measurements of tension. The principle of the gas measurements is the same as that commonly employed in microrespirometric work, namely, the observation of the contraction in volume in a chamber containing the tissue, together with a little alkali, whose function is to keep the carbon dioxide tension negligible. To record the tension, a fine wire is led from the upper end of the muscle through a fine capillary plugged with vaseline. This vaseline seal appears to be quite gastight and to cause no measurable error in the gas measurements.

The apparatus consists essentially of two suitably shaped glass vessels (*A* the respiration, and *B* the compensation, chamber) in communication with a capillary manometer showing the pressure difference between the chambers: together with a calibrated gas burette by means of which the necessary measured volume of gas may be forced into the respiration chamber to bring the manometer to equality on the two sides. Readings of the burette give directly the volume of gas absorbed by the tissue, the correction for pressure and temperature being that for the conditions prevailing at the moment of closing the apparatus.

The instrument thus consists essentially of a Barcroft differential manometer with the addition of the burette of the Winterstein microrespirometer [Winterstein, 1912]. The use of the burette has several advantages; that calibration of the chamber volume is unnecessary, that the pressure may be maintained constant in the respiration chamber during a prolonged experiment, and that the range of the instrument is increased, so that greater changes of volume may be measured than with a simple differential manometer of equal sensitiveness. The manometer was adopted instead of the oil drop in a horizontal tube used by Winterstein, Thunberg and others, since it possesses a much greater range for equal compactness, and it should not be subject to the curious spontaneous movements described by some who have used the latter arrangement [Winterstein, 1912; Haberlandt, 1911].

[1] This work was carried out during the tenure of the Benn W. Levy studentship.

Details of construction.

The scale of centimetres in *D* gives the scale of the drawing (Fig. 1). The respiration chamber (*A*) has an opening at the bottom, closed with a ground stopper, through which the tissue is introduced. One platinum electrode passes through the stopper, which is continued in the form of a bent tube reaching above the level to which the instrument is immersed in the water-bath. This tube is filled with salt solution and serves to conduct the stimulating current to the muscle without short-circuiting through the water-bath. There is space at the bottom of each chamber for about 1 cc. of the CO_2 absorbent. Each chamber is provided with a tube and tap, which serves as an exit for the gas when filling. The compensation chamber (*B*) is made of similar dimensions to the other, but is not provided with the opening at the bottom nor with the capillary at the top. Exact correspondence in volume is unnecessary, but the chamber walls should be of the same thickness.

The chambers are connected with the manometer and burette by accurately ground joints, and are held in place by elastic bands passing over the hooks shown.

The manometer and burette, *C* and *D* (Fig. 1), with the necessary connections for filling, are made in one piece of glass and are clamped upon a glass scale mounted on a board: for this purpose ordinary spring wooden clothes-pegs have been used. The manometer is of about $\frac{1}{2}$ mm. bore and is fused at each end to the three-way stopcocks 1 and 2. The burette and its connections have the form shown in the drawing. The portion 4–5–6 is calibrated before being built into the instrument: it is provided with one mark at 5 on the glass which gives a means of fixing the burette during an experiment over the same part of the scale as at the time of calibration: a precaution which is only necessary if the calibration shows that the inequalities are great enough to affect the accuracy of an experiment. Below 6, the calibrated tube is fused to a bent tube ending in a thick walled rubber tube of suitable length (three to five inches according to bore), closed with a glass stopper at the top and provided with a number of ordinary spring pinch clips for adjusting the mercury level. A stopcock, 3, is fused on to the burette at the top for filling purposes.

The manometer with its taps, and the burette with its connections, may be made and fitted to the board separately, then clamped to the board in their correct positions over the scale and the final joint made by fusing the glass at 7 when the parts are on the board. It is well to strengthen the apparatus by securing the mercury tube to the manometer at the point 8 where the tubes cross.

Cleaning and filling.

All parts of the apparatus are filled with a chromic acid solution and the whole is either immersed in, or heated over (if a sufficiently large basin is not available), a basin of the same fluid. Frequently many hours' heating at or near the boiling point is necessary. The acid is washed out, finally using

distilled water, and at this stage insufficient cleaning may be detected by the presence of greasy unwetted places on the glass. Drying is simply effected by warming on or over a sand-bath while air is drawn through all parts of the apparatus. Care must be taken that all parts are thoroughly dried in turn.

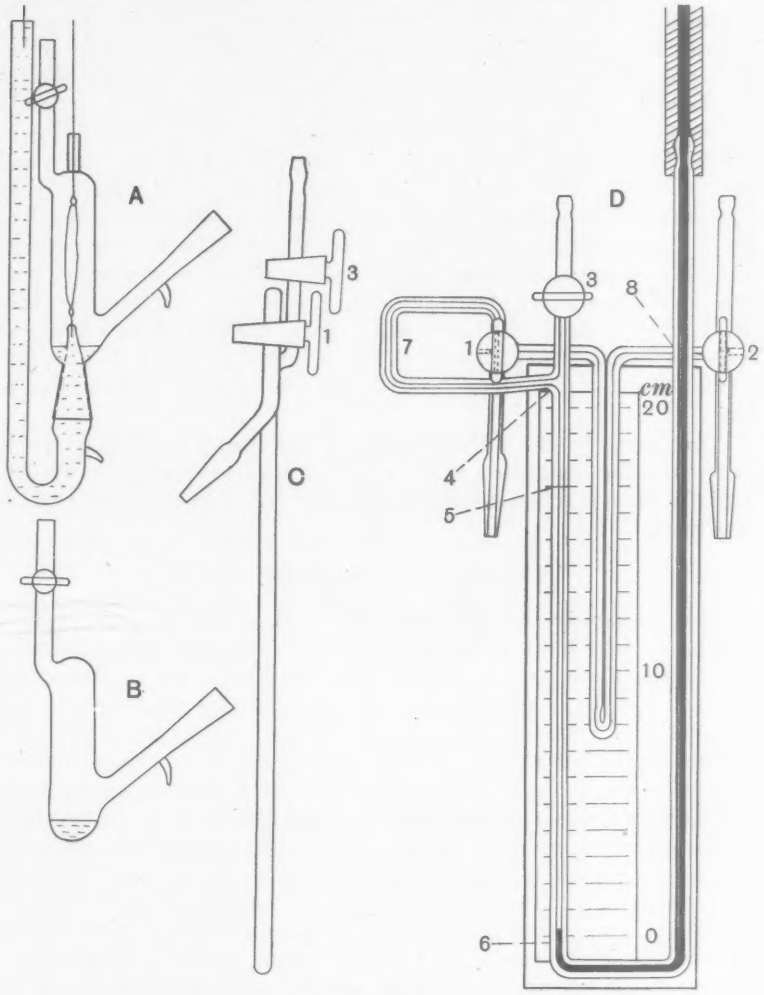

Fig. 1.

The tap grease employed was a mixture of white vaseline and paraffin wax (2 : 1) and was used as sparingly as possible: this was selected in preference to the usual lubricants on account of its smaller power of absorbing oxygen.

Filling the manometer.

Paraffin oil boiling between 160° and 190° approximately is suitable, having a low vapour pressure and high mobility, so that equilibrium is reached at once. Clove oil, which is occasionally recommended for capillary manometers, is much too viscous. The barrel of the tap, 1 (or 2), is greased and inserted: the apparatus is held in a stand with the tap, 2 (or 1), from which the barrel has been removed, above the other, and a small amount of oil is introduced in a pool above the opening of the manometer: by cautiously opening the lower tap the correct quantity is allowed to run in, avoiding air bubbles. The excess in the tap seating is cleaned away with filter paper, and the column allowed to run down into place. It should fill the two limbs slightly more than half full. No difficulty is found in adjusting the length of the oil column if it is run back again to the empty tap and excess removed with filter paper or more added with a capillary pipette as may be required. Taps 2 (or 1) and 3 may now be inserted. Except when the instrument is in use it is well to leave both taps 1 and 2 closing the ends of the manometer (as in the diagram), since if the oil column is broken or allowed to come into contact with the tap grease the apparatus will probably require cleaning again.

The mercury burette is filled from the rubber tube: it is well to fill it only to within a few centimetres of the zero mark, since the mercury in the long vertical column may descend slightly, owing to the contraction of the rubber or to leaks.

Details of manipulation.

The compensation chamber, containing a little potash (2½ %), is attached. The excised tissue is tied to the platinum loop on the stopper of the respiration chamber and the other end to the fine wire: the wire is threaded through the capillary, and the capillary dipped into molten vaseline till the grease runs up to within about 2 mm. of the chamber proper. It must not be allowed to spread over the inside of the chamber or errors due to the grease absorbing oxygen will occur. Finally about 0·5 cc. of the potash is introduced by means of a capillary pipette and the respiration chamber attached to the remainder of the apparatus, which is placed in a well-stirred water-bath, preferably provided with a thermostat. The taps on the chambers are opened and the gas mixture required is passed in through taps 2 and 3 and out through the chambers. If the mercury in the burette is raised to the level of the T-piece when the gas is first passed, and is quickly lowered to the zero before discontinuing the stream, the whole of the apparatus is washed out except the manometer, which is a negligible volume. The incoming gas stream is then shut off at 2 and 3, tap 2 turned so as to connect the compensation chamber with the manometer (through 90° clockwise from the position of the diagram), tap 1 to connect the respiration chamber with the manometer and burette

(through 180°), and the taps on the chambers closed. The barometer and temperature are read.

Reliable readings can usually be taken 15 minutes after closing the taps. To take a reading, the screw clips are opened or closed so as to alter the mercury level until the manometer shows equal pressures in the respiration and compensation chambers. The height of the mercury is then read, parallax being avoided by the silvering on the back of the glass scale. When the mercury reaches the top of the calibrated tube the gas must be renewed, and the mercury adjusted to zero during the passage of the gas; this need not interrupt the readings for more than 20 minutes. With my two instruments the volume of one centimetre on the burette is 0·02266 and 0·01231 cc. respectively, so that the length of 20 cm. suffices for several hours' continuous readings at a rate of respiration of the order 0·03 cc. per hour.

Limits of accuracy.

The stability of the zero of this instrument is the most important factor determining the accuracy with which rates of oxygen absorption can be measured. In blank experiments without any tissue but with the potash in the bulbs, it has been found that, neglecting the first 20 or 30 minutes after passing the gas, the variations in zero are appreciable though small, not usually exceeding 0·5 cubic millimetres per hour for the first hour, and falling considerably later. The cause of these variations has not been definitely ascertained; they may be due to absorption of gas by the grease used for the joints and taps, or possibly to very slight movements at the ground joints. In any case the minimum possible amount of tap grease should be employed and this should consist of substances as saturated chemically as possible, consistent with proper lubrication of the surfaces. The figures are given for the approximate amount of zero change I have observed with the grease made of paraffin wax and white vaseline: mixtures containing rubber, and ordinary vaseline, appear to give a much larger spontaneous change. An apparatus similar in principle to this, but with joints of rubber pressure tubing instead of ground joints, was found to have a very much larger zero change, comparable in amount with the actual consumption of small muscles, and since this tendency to change zero was either abolished or much reduced when the apparatus was filled with hydrogen instead of oxygen, it appears to be due to the absorption of oxygen by the rubber connections. The errors with the present apparatus are of the order 1 in 20,000 of the volume of the chambers: it may be of interest to note that I have observed similar slow changes of the zero of an ordinary Barcroft blood gas apparatus.

The oxygen consumption of a single resting sartorius of the frog is of the order 20 cubic millimetres per hour, so that this quantity may be determined to an accuracy of 2 or 3 %.

Errors due to a possible imperfect absorption of CO_2 appear to be very small. During a period of constant respiration the apparatus will reach a

steady state, and any small amount of CO_2 unabsorbed will remain constant so that no error is introduced if the volume diminution is taken as the oxygen absorption. When a change of rate of respiration, say an increase, occurs, there will be a slight accumulation of unabsorbed CO_2, so that the diminution in volume will be less than the true absorption of oxygen. The amount of the increase in the unabsorbed CO_2 will be proportional to the increase in the rate of production of CO_2, and may be calculated approximately from the dimensions of the apparatus and the constant of diffusion of CO_2 into air. The increase in the quantity of CO_2 escaping absorption which must be present in order to raise the concentration gradient of CO_2 between tissue and absorbent till the steady state is again reached at the increased rate of production, for an increase in rate of CO_2 production of 1 cubic millimetre per minute, may be calculated to be as follows. It is assumed for simplicity that the chambers are cylindrical and that the CO_2 is all evolved at the same distance from the absorbent (column 1). Column 2 gives the amount of the increase in the volume of unabsorbed CO_2 in cubic millimetres.

Distance cm.	Total increase of unabsorbed CO_2 cmm.
1	0·05
2	0·2
2·5	0·3
3	0·45
3·5	0·6
4	0·8

These amounts are several times larger than any at all likely to be met with, since the assumed rate of increase in the CO_2 production is several times larger than is probable. It is clear that the difference between the volume diminution and the oxygen absorption is so small as to be usually within the error of reading the scale with the naked eye, and is negligible unless extremely rapid changes of rate are to be followed with great accuracy.

A third condition, the effect of which must be considered, is the progressive change in the composition of the gas in the respiration chamber, for this will have an effect on the rate of respiration of the tissue, for which allowance may have to be made. The chambers used have a volume of about 10 cc., so that the absorption of 0·05 cc. of oxygen (a probable amount for work with large muscles in one hour) will cause a diminution of the oxygen concentration of about 0·5 %. A diminution in the volume corresponding to 20 cm. of the larger burette mentioned will reduce the oxygen tension by 4·5 % of an atmosphere. In cases where this alteration might have an important effect, larger chambers would be required, as recommended by Krogh [1915, p. 526].

SUMMARY.

An instrument, modified from Barcroft's blood gas apparatus and Winterstein's microrespirometer, is described, for determining the oxygen consumption of frog's muscle or other small amounts of tissue in any desired atmosphere. The instrument permits of simultaneous observations on the tension of contraction.

I am indebted to the Royal Society for a grant covering the cost of this work.

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LXIV. THE FERMENTATION OF CELLULOSE IN THE PAUNCH OF THE OX AND ITS SIGNIFICANCE IN METABOLISM EXPERIMENTS.

By AUGUST KROGH and HANS OLUF SCHMIT-JENSEN.

From the Laboratory of Zoophysiology, University of Copenhagen.

(*Received September 25th, 1920.*)

It is well known that in herbivorous animals and especially in ruminants the fermentation processes taking place in the gut are so intensive that they must be taken into account in metabolism experiments if serious errors are to be avoided, but the best methods of measuring the fermentation and of utilising the measurements for correcting the energy balance are still matters of doubt and controversy, though they have been the object of several investigations. We have therefore made a series of experiments which, though they do not solve the problem, will at least make it a little less obscure.

It has long been recognised that the fermentation in the paunch of the ox is mainly a methane fermentation of cellulose. The value of the fermentation from the point of view of the animal's economy lies in the fact, that the wholly indigestible cellulose is converted into substances which can be absorbed and utilised, but this gain is obtained only at the cost of a certain proportion of the energy available in the cellulose. The problem in which we are interested can be formulated as the determination of the loss of energy involved in the fermentation, though the way in which we propose to attack it is an indirect one.

It was shown by Markoff [1913] that in the fermentation cellulose is split up into fatty acids of the average composition of butyric acid, carbon dioxide and methane. From many of Markoff's experiments small quantities of hydrogen are also reported as being present, but their reality appears to us to be extremely doubtful. In many metabolism experiments on cattle [H. Möllgaard and A. C. Andersen, 1917; A. C. Andersen, 1920] and in all our own experiments on the fermentation of material from the paunch no hydrogen was produced, and we must point out that the routine of combustion analysis used in Zuntz's laboratory and adopted by Markoff is very liable to show positive, but spurious hydrogen percentages[1]. We are convinced,

[1] The combustion of methane requires a comparatively high temperature, but if the platinum wire in the combustion pipette is too hot methane is dissociated into carbon and hydrogen, and part of the carbon will generally escape combustion. The contraction upon combustion will in such a case be more than double the volume of carbon dioxide formed, and the surplus is set down as hydrogen.

therefore, that hydrogen is not a product of the cellulose fermentation in the paunch.

When the products formed by the fermentation are always the same, viz. CO_2, CH_4 and $C_4H_8O_2$, there is reason to believe that we have to do with a definite reaction and constant proportions between the products. The reaction might be imagined for instance to take place according to the equation

$$2C_6H_{10}O_5 = 2C_4H_8O_2 + 3CO_2 + CH_4$$

involving a proportion between the volumes of CO_2 and CH_4 produced as 3/1. The chief object of our experiments has been to study the proportion between the CO_2 and CH_4 formed by the fermentation, to find out whether it is constant or not, and to determine it numerically.

Such a study has been undertaken before by Boycott and Damant [1907] and especially by Markoff [1913], but the results were very disappointing, the relation $\frac{CO_2}{CH_4}$ being extremely variable and almost always very high. It can be shown, however, that this is to a great extent due to methodical errors. The fermenting mass in the paunch contains large quantities of carbonates and bicarbonates, especially from the saliva with which the fodder is mixed during rumination. The fatty acids formed will liberate CO_2 from the carbonates, and unless means are found to determine separately the CO_2 formed by the fermentation from organic sources, the ratio of CO_2 to CH_4 will be higher than that demanded by the equation of the reaction. Boycott and Damant made no attempt to distinguish between fermentation CO_2 and carbonate CO_2. Markoff tried to determine the carbonate separately, but the methods adopted were not sufficiently accurate[1], and nothing definite is known therefore regarding the proportion of CO_2 formed by the fermentation to the CH_4.

Methods.

We have used the following technique.

The samples of material were collected by one of us in the Copenhagen public slaughter-houses immediately after the animals had been killed. The cows slaughtered had been fed for one or more days on hay and straw only, and we were likely therefore to find the cellulose fermentation comparatively unmixed with other fermentation processes which might take place in the paunch. In some cases the samples were carried to the laboratory in Dewar flasks, so as to maintain their temperature, but usually they were allowed to cool, as this did not apparently make any difference. In the laboratory the samples were mixed with such an amount either of water or of a suitable buffer mixture ($\frac{1}{7}$ molar phosphates) that it became possible to take out small representative samples. Generally the larger particles of straw had to

[1] In most experiments the samples were sterilised by boiling before the CO_2 was determined. As the contents of the paunch have a high CO_2 tension a very considerable loss could not possibly be avoided.

be removed before taking samples. After thorough stirring, and while stirring was still going on, the samples were taken by means of a small vessel (30 cc. crucible) which was dipped into the mixture. The samples were transferred to fermentation flasks of about 50 cc. capacity, and the exact quantity taken determined by weighing.

The fermentation flasks were provided with a side tube reaching down to the bottom of the vessel and closed by means of a tap. (See Fig. 4, no. 1, in the paper by Krogh [1914, 2].) After weighing, each fermentation flask was connected with a manometer of the type used for microrespiration (Fig. 1, Krogh [1914, 2]), but with somewhat wider tubes, and filled with mercury instead of oil, and put into a water-bath at body temperature, in which arrangements for shaking them vigorously were provided. Before the fermentation experiment could begin, the air in the flasks was washed out by a current of pure nitrogen[1]. About 100 cc. of nitrogen were introduced through the side tube, and after bubbling through the mixture taken out through the top of the manometer and collected over mercury in a gas receiver. During this process, which occupied 5–10 minutes, the bottles were shaken vigorously to establish equilibrium between the gas current and the fermenting mixture and especially to drive out all the oxygen. Finally a slight surplus pressure was established, the tap on the side tube closed, the shaking continued for one or two minutes more, a sample of the gas drawn out at the top of the manometer for subsequent micro-analysis [Schmit-Jensen, 1920][2], the manometer closed, read and noted together with the barometer, temperature of bath and time of beginning.

The fermentation could now be followed on the manometer, and when a suitable pressure had been reached, the flasks were again vigorously shaken. Immediately afterwards the manometer was read[3] and recorded together with the time and the temperature of the water-bath, whereupon a sample of the air in the flask was collected after washing out the manometer tubing by means of part of the excess pressure.

After the sampling of the gas 2 cc. of 20 % hydrochloric acid were introduced through the side tube to stop fermentation and liberate the CO_2 from the mixture, and while a vigorous shaking was kept up, the CO_2 was driven off by a current of air, taken up in baryta solution and determined by titration as described by Krogh [1914, 1]. For the air current the nitrogen (+ CO_2)

[1] We have used nitrogen from air in a gasometer with pyrogallate solution. The generator described by C. van Brunt [1914] is certainly much better.

[2] This analysis is necessary, because the fermenting mixture always possesses a more or less considerable CO_2 tension. In some cases a trace of oxygen has been left, and in others a slight amount of methane was also present.

[3] While the fermentation flask is in a water-bath at 37° moisture is apt to distil up into the narrow tubes of the manometer and collect there in short columns of water, which by their capillary resistance prevent the uniform movement of the column of mercury. To prevent this the fermentation flask was in the later experiments shut off by a clip from the manometer during the whole of the experiment, and connection established only when it was desired to take a reading.

stored since the beginning of the experiment was first used and afterwards supplemented by a suitable amount of CO_2-free air. In this manner the baryta would absorb all the free and combined CO_2 present in the fermenting mixture before fermentation + the quantity formed during fermentation − the minute quantities present in the gas samples. These were added as a correction.

The quantity of CO_2 present before fermentation was determined in the same manner on separate samples treated just as the others, put into fermentation bottles and placed in the water-bath, but acidified with HCl immediately afterwards.

The gas samples taken at the beginning and end of the fermentation experiment were analysed by the micro-method worked out by Schmit-Jensen [1920] for CO_2, H_2, CH_4 and occasionally O_2, while the remainder was taken to be nitrogen. From the analyses and the pressures and temperatures, the quantity of each gas at the beginning and end of the fermentation was calculated.

We reproduce as an example the protocol and calculation of Exp. 8 B.

Material. From paunch of a single cow, fresh contents of brownish yellow colour, on the whole finely triturated, but containing straw particles of about 3 cm., rather dry, not foaming, alkaline towards litmus, acid to phenolphthalein.

Emulsions for experiments: A. 250 g. material + 500 cc. secondary phosphate ($\frac{1}{7}$ molar).
B. 250 g. + 500 cc. phosphate (7 primary, 3 secondary).

Specific gravity of emulsion 1·034.

Sample B. 22·88 g. = 22·14 cc., B^1 (for CO_2 determination) 23·81 g., B^2 23·81 g.

CO_2 determinations:

B^1 5·02 cc. or 0·211 per g., B^2 4·76 cc. or 0·200 per g. Average per g. 0·206 cc. B after fermentation 11·83 cc. According to the average of B^1 and B^2 it should have contained 4·72 cc. before fermentation. CO_2 produced by fermentation therefore 7·11 cc.

Volume of fermentation flask B

59·26 cc. + manometer tubing 0·27 cc. − glass beads for shaking 0·20 cc.

Volume of 1 mm. manometer tubing 0·0009 cc. Volume of compensating bottle 19·95 cc.

Manometer readings.

19′	4^{30} p.m.	138·3	142·8	Diff. d_4=4·5. T. 37·0°. Barometer, 733 mm.
	8^{00} p.m.	122·5	159·0	
	10^{35} p.m.	115·0	166·5	
20′	11^{00} a.m.	101·0	182·0	
	1^{30} p.m.	97·5	186·0	
21′	12^{00} m.d.	83·2	200·5	Diff. d_7=117·3. T. 37·5°.

Analyses (corrected for systematic error).

	Initial sample		Final sample		Average	
%	1	2	1	2	Initial	Final
CO_2	2·0	2·1	5·4	5·5	2·05	5·45
O_2	0	—	0	—	0	0
H_2	—	—	—	0	0	0
CH_4	0	—	8·1	8·4	0	8·25
N_2	—	—	—	—	97·95	86·3

The total quantity of gas in the apparatus at the beginning and end of the experiments has been calculated as follows: at the beginning we have the gas volume $A = 59{\cdot}26 + 0{\cdot}27 - 0{\cdot}20 - 22{\cdot}14 = 37{\cdot}19$ cc. By reducing this to 0° and 760 mm. we get

$$A\,\frac{273}{273+t_w}\cdot\frac{P-f_w+d_i}{760}=37{\cdot}19\,\frac{273}{273+37{\cdot}0}\cdot\frac{733-46{\cdot}5+4{\cdot}5}{760}=29{\cdot}75\text{ cc.}$$

At the end of the experiment we have the total gas volume

$$A_1=A+\frac{A+C}{2C}\,vd_f=37{\cdot}19+\frac{37{\cdot}19+19{\cdot}95}{2.19{\cdot}95}\,0{\cdot}0009\times117{\cdot}3=37{\cdot}19+0{\cdot}15=37{\cdot}34$$

and reduced to 0° and 760 mm.

$$A_1\,\frac{273}{273+t_{w_1}}\cdot\frac{P-f_{w_1}+d_f}{760}=37{\cdot}34\,\frac{273}{273+37{\cdot}5}\cdot\frac{733-48+117{\cdot}3}{760}=34{\cdot}67.$$

The initial and final quantities of each gas are found by multiplying the volumes with the corresponding percentages.

cc.	Initial	Final	Produced	
CO_2	(0·61)	(1·89)	7·11	Relation $\frac{CO_2}{CH_4}=\frac{7{\cdot}11}{2{\cdot}86}=2{\cdot}49$
CH_4	0	2·86	2·86	
N_2	29·15	29·9	0·75	

Accuracy and sources of error. The accuracy of our determinations is not very great. It is very difficult to obtain good average samples of materials like the contents of the paunch, and the method finally adopted is the outcome of a number of trials. Even this method, however, is not so accurate as we should desire.

The samples for determining the initial quantity of CO_2 have always been taken and analysed at least in duplicate. Special trials have shown that our CO_2 determinations are not vitiated by fatty acids or HCl being driven over into the baryta. A statistical treatment of the determinations shows that the dispersion of the results from single samples is ± 3 % with a maximum of 5 %. The differences between the initial and final quantities of CO_2, which are affected by these errors, are of the same order of magnitude as the initial quantities themselves, and the mean error on the determination of CO_2 production is in most experiments about ± 4 %.

The gas analyses have been corrected for the systematic errors found by Schmit-Jensen. According to the double determinations made in the course of the present investigation, the mean error of a double determination of CH_4 works out as ± 0·12 %. As the average CH_4 percentage in the samples

is about 7, the percentage error on the quantity of CH_4 works out as 1·7 %. There is a further slight error, due to the determination of total pressure. The mean error on the quantity of CH_4 is therefore about 2 %.

The relation $\frac{CO_2}{CH_4}$, which has been the main object of our determinations, is generally found to lie about 2·5. It will of course be influenced by the errors on the CO_2 and CH_4 determinations, and the resulting mean error can be calculated. We have

$$\frac{CO_2}{CH_4} = \frac{2{\cdot}5 \pm 0{\cdot}1}{1 \pm 0{\cdot}02}$$

and hence the error

$$\pm \sqrt{2{\cdot}5^2 \times 0{\cdot}02^2 + 0{\cdot}1^2} = \pm \sqrt{0{\cdot}0125} = \pm 0{\cdot}11.$$

The determination of total gas pressure has in most of the experiments been rather inaccurate, because drops of condensed water obstructed the tubing between the flasks and the manometer. The error may have amounted to 4 or even 6 mm. or 0·5 to 0·8 % of the total pressure. Such an error has very little influence upon the determination of CH_4, because the percentage of this gas is always small, but the influence on the nitrogen determination is considerable, and as this determination is further influenced by the sum of the gas analytical errors, the mean error on the difference between the initial and final quantity of nitrogen becomes considerable, viz. about ± 0·25 cc.

Experimental Results.

The results of our experiments are summarised in Table I.

The constancy of the gaseous nitrogen. In Exps. 1, 2, 3, 4, 6 and 7, the gaseous nitrogen remains constant within the limits of error. The average production in one of these experiments is − 0·06 ± 0·18 cc. In Exp. 8 A we have no determination of the nitrogen, and the production of 0·75 cc. found in 8 B is probably due to some undetected error. In Exps. 5 A and B we have observed a slight, but probably real, production of gaseous nitrogen. Krogh [1916, p. 53] has found that the presence of nitrates in the fermenting contents of the coecum of rabbits will cause a liberation of free nitrogen. Nitrates or nitrites may, perhaps, have been present in the paunch in this case, though we failed to detect macroscopic remains of beet root, which contains considerable quantities of nitrate[1]. In any case we can conclude from our experiments that free nitrogen is neither produced nor absorbed in appreciable amount by the fermentation of cellulose in the paunch.

The production of methane. In column 6 of Table I we have given the average rate at which methane has been produced. This has been figured out per 24 hours and for 100 kg. of undiluted material from the paunch in order to facilitate comparison with the quantities of methane produced in metabolism

[1] According to determinations made in the Danish "Forsögslaboratorium" beet root may contain from 0·05 to 0·15 g. HNO_3 in 100 g. fresh substance. Turnips do not contain any nitrates.

Table I.

No.	Material		Emulsion in	Duration of exp. hours	Nitrogen produced cc.	CH_4 produced per 100 kg. in 24 h.	Rate of ferment. mm. per hour 1st day	2nd day	3rd day	$\frac{CO_2}{CH_4}$
1	Contents of paunch, finely triturated	A	Secondary phosphate	24	0	54	7, 3·5, 3·5	—	—	2·85
		B	Water	5·5	−0·2	79	18, 19, 17	—	—	2·95
2	Fermentation in paunch very strong. Material moist, fairly well triturated, alkaline to litmus, acid to phenolphthalein	A	Secondary phosphate	25	−0·2	19	2·3, 2	—	—	2·7
		B	Secondary phosphate	25	0	18	2·3, 2·4	—	—	2·7
3	Fermentation not very pronounced. Very finely triturated, alkaline to litmus, acid to p.p. Used after 6 hours		7 sec. phosph. + 3 prim. phosph.	14	0	63	10, 17	—	—	2·95
4	Fermentation rather weak. Coarse, straw and turnip. Neutral to litmus	A	7 sec. phosph. + 3 prim. phosph.	17	−0·2	74	12, 8, 11	—	—	2·2
		B	7 sec. phosph. + 3 prim. phosph.	15·5	0	75	6, 10, 25	—	—	2·2
5	Fermentation weak. Finely triturated, very moist. Alkaline to litmus, acid to p.p.	A	7 sec. phosph. + 3 prim. phosph.	63	+0·6	9	1·9, 2·1	1·1	0·5	(2·8)
		B	7 sec. phosph. + 3 prim. phosph.	63	+0·6	10	2·7, 2·2	0·8	0·7	(2·2)
6	From 5 animals, rich in larger particles, alkaline to litmus, acid to p.p.	A	Water	23	+0·1	28	8, 6, 8	—	—	2·4
		B	Water	24	−0·4	28	7, 6, 7	—	—	2·4
7	From 13 animals, coarse moist, contains turnips. Turns p.p. slightly red. Used after 6 hours	A	Water + $CaCO_3$	65	0	6	2·2	—	1·2	(4·9)
		B	Water + $CaCO_3$	66	+0·2	6	2·1	—	1·2	(4·0)
8	From single animal. Fermentation weak. Finely triturated, rather dry. Alkaline to litmus, acid to p.p.	A	Secondary phosphate	45	?	17	6, 5, 2	1·4	—	2·65
		B	7 sec. phosph. + 3 prim. phosph.	43	+0·7	21	8, 6, 2·5	1·2	—	2·5

experiments on cattle. Since a cow, the paunch of which contains between 50 and 100 kg. of food, will produce from 100 to 300 l. methane in 24 hours, it is evident that the intensity of the fermentation has even in the best of our experiments been considerably below that observed in natural conditions, and in most of our determinations it has fallen far short of this ideal. In Markoff's experiments the intensity of fermentation has also, generally, been lower than in the living animal. This is ascribed to the cooling and to the influence of oxygen in the interval between the taking out of the material and removal of the oxygen in the water-bath at 37°. We believe that the contact with oxygen is the factor chiefly responsible.

In column 7 we have given some figures indicating roughly the variation in fermentation rate in the course of each experiment. The figures give the increase in pressure in the fermentation flasks in mm. mercury per hour. They are comparable only within the same experiment and to a certain extent in experiments made with the same buffer mixture. When, for instance, the rate is much higher in Exp. 1 B than in 1 A, this means only that far more CO_2 is evolved as gas in B than in A, owing to the greater acidity of the fermenting mixture.

In Exps. 1 B, 2, 4 A, 5, 6 and 7 the rate remains practically constant during the first 24 hours. In 1 A and 8 it decreases, and in 3 and 4 B it increases materially. In Exps. 5, 7 and 8, which have been continued beyond 24 hours, the rate has decreased on the second day and still more on the third. We are unable to give any explanation of the variations in rate.

The relation $\frac{CO_2}{CH_4}$ given in the last column varies between 2·95 and 2·2, except in Exp. 7, in which the fermentation was extremely slow and the $CaCO_3$ added had probably made it abnormal. Except in this case it does not seem to be in any way influenced by the varying intensity of the fermentation. The double determinations agree very closely, except in the two experiments 5 and 7 lasting over 48 hours and showing the slowest fermentation. The differences between different experiments are so much larger than the mean error of a determination that they must be considered as real, and it must be admitted, therefore, that in the conditions obtaining the relation $\frac{CO_2}{CH_4}$ is *not* constant, though the variations found are very much smaller than formerly supposed.

The experiments show that within the range of hydrogen ion concentrations which have been found in the paunches of cows (P_H between 7 and 8) the reaction of the fermenting mixture has no influence upon the character or intensity of the fermentation.

The average for the experiments 1, 3 and 4, which show a fairly rapid rate of fermentation, is $\frac{CO_2}{CH_4} = 2{\cdot}6$, and the average for all the experiments, except the abnormal nos. 5 and 7, is likewise $2{\cdot}6 \pm 0{\cdot}08$ with a dispersion of $\pm 0{\cdot}27$.

The application of the result in metabolism experiments on cattle.

The figure 2·6 can be utilised to correct the results directly derived from determinations of the gaseous metabolism. A certain caution is necessary, however, since the relation $\frac{CO_2}{CH_4}$ is not quite constant, and may possibly show larger variations in cows fed upon a more varied fodder.

To illustrate the application of our result, we have taken an experiment made by A. C. Andersen [1920], in which both the gas exchange and all solid ingesta and excreta were determined and calculated on a 24 hour basis. The metabolism of nitrogen-free substances was found to require 1451 l. oxygen and produce 1583 l. CO_2 and 127 l. CH_4. The apparent R.Q. is $\frac{1583}{1451} = 1{\cdot}091$. According to our result $2{\cdot}6 \times 127 = 330$ l. CO_2 should be due to fermentation and the true R.Q. for the organism itself should be $\frac{1583 - 330}{1451} = 0{\cdot}864$.

The energy metabolism is calculated from the oxygen intake by means of Zuntz's formula. We have

$$E = O_2 \{4{\cdot}686 + (\text{R.Q.} - 0{\cdot}707)\, 1{\cdot}23\}.$$

In the present instance the uncorrected heat production would be $1451\ (4{\cdot}686 + 0{\cdot}384 \times 1{\cdot}23) = 7487$ Cal. and the corrected only 7079 Cal.

The summary calculation is evidently inadmissible. Against the application of the correction it might be argued that Zuntz's formula has been elaborated on the assumption of a metabolism of ordinary fats and carbohydrates only, and cannot be used when the lower fatty acids formed by the fermentation are catabolised. If Markoff is right, that the fatty acids correspond on an average to butyric acid, the caloric value of the oxygen used for the catabolisation of these substances will correspond closely to that obtained from the formula. Butyric acid has a respiratory quotient of 0·8 with a caloric value of the oxygen of 4·74 Cal. per l., while the formula gives 4·80.

It is of special interest to compare our calculation with the elegant solution of the problem given by A. C. Andersen himself. Andersen's reasoning is as follows: if the methane produced by the fermentation is burned the total (non-protein) metabolism (methane included) is one of carbohydrate and fat, and Zuntz's formula must be strictly applicable to find a total heat production, and if the heat of combustion of the methane is afterwards subtracted, the rest is the metabolism of the animal.

Methane burns according to the equation $CH_4 + 2O_2 = CO_2 + 2H_2O + 213$ Cal. The volume of oxygen used is double that of the methane and carbon dioxide, and we have consequently in the above example

Total $CO_2 = 1583 + 127 = 1710$ l. Total $O_2 = 1451 + (2 \times 127) = 1705$ l. R.Q. = 1·003. Hence the total heat production 8610 Cal. and, by subtraction of the heat of combustion of 127 l. methane = 1207 Cal., the metabolism of the animal itself 7403 Cal.

This figure is 324 Cal. or 4·6 % higher than that obtained by our calculation. That it should be higher was to be expected, since in Andersen's calcula-

tion the amount of heat produced by the fermentation in the paunch is included, while our calculation aims at having it excluded. The heat of fermentation cannot strictly be reckoned as due to the metabolism of the animal and is practically lost from the point of view of the animal's economy, even though it is generated inside its body[1].

According to the provisional formula of fermentation given above (p. 687) the heat of fermentation per g.-molecule of methane would be equal to the heat of combustion of 2 g.-molecules of $C_6H_{10}O_5$ (1356 Cal.) minus the heat of combustion of 2 g.-molecules of $C_4H_8O_2$ (1054 Cal.) and 1 of methane (213 Cal.), that is 89 Cal. or 3·97 Cal. per l. methane. In the experiment under discussion the loss by fermentation would therefore, on this assumption, amount to 505 Cal. When Andersen's figure is reduced by this amount it becomes 6898 Cal., 181 Cal. or 2·6 % lower than ours.

Our experiments show that the provisional formula does not represent the fermentation exactly, and it is probable, moreover, that part of the energy liberated by the fermentation is utilised for synthetic processes (building up of organisms?) within the fermenting mixture. The loss is therefore probably somewhat lower than indicated by the formula.

It would be desirable to get direct bio-calorimetric determinations on the fermenting mass combined with determinations of the CH_4 evolved. The making of such determinations has been attempted by Markoff [1913, p. 63], but so far as we are aware the results have not been published. We have also made some attempts, but have not been able to overcome satisfactorily the technical difficulties. So far as they go, our experiments indicate a heat of fermentation of about 50 Cal. per g.-mol. of CH_4. When corrected by means of this figure Andersen's results would become 7119 Cal., 40 Cal. or 0·6 % higher than ours. The closeness of this agreement has of course no real significance, but it would seem, nevertheless, that our method of calculating the energy expenditure of the ruminating organism itself cannot be very far wrong.

Summary.

Methods are described for measuring the gaseous products of fermentation processes and especially the CO_2 and CH_4 evolved by the cellulose fermentation taking place in the paunch of the ox.

Usually gaseous nitrogen is neither absorbed nor produced by this fermentation.

The relation between the CO_2 and CH_4 formed by the fermentation appears to be independent of the rate of fermentation and, within the limits investigated, of the hydrogen ion concentration. The relation $\frac{CO_2}{CH_4}$ works out

[1] In the calculation made by Andersen it is perfectly legitimate to include this amount of heat, since he compared the heat production found by respiration experiments (+urine analysis) with the heat computed from weighings and analysis by means of the calorimetric bomb of all the ingesta and excreta of the animal.

as on an average 2·6, but real variations occur. The limits observed are 2·2 and 2·9 respectively.

A method is suggested by which the observed respiratory exchange of cattle can be corrected, when the production of methane is measured.

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LXV. A METHOD FOR OBTAINING UNCONTAMINATED SPECIMENS OF URINE FROM THE BILLY GOAT; WITH SOME NOTES UPON THE NORMAL METABOLISM OF THIS ANIMAL.

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The method described in this communication[1] was applied during the war to a study of the normal urine of the goat. By its means some data upon the normal metabolism of the goat were collected, and although by no means complete, it has been thought worth while to put them upon record as they are in general agreement with the work of others. Steenbock, Nelson and Hart [1914] showed that the calf used ammonia for the purpose of neutralising acid given in the diet. Winterberg [1898] had previously shown that the rabbit used ammonia under similar circumstances. Some previous observations upon the goat have been made by Baer [1906], who showed that under conditions of starvation, the output of ammonia was increased.

The notes dealt with in this paper are concerned with the urine of the goat upon different diets, during starvation, and after feeding with acid and alkali. For this purpose the titratable alkalinity of the urine and the hydrogen ion concentration have been studied, as well as the ammonia excretion, and in some cases the excretion of chlorides. The reaction of the urine is expressed throughout in the usual Sörensen notation. For the estimation of the titratable alkalinity, $P_H = 7{\cdot}45$ has been used as the titration end point.

I. Methods.

For the preservation of the samples of urine, chloroform and toluene were used. No precautions were taken to avoid loss of CO_2 during the collection of the samples.

1. *The Collection of Samples of Urine.* A crate of design and dimensions shown in Fig. 1, made of wood, was used for the goat in all experiments. It has a three-inch zinc lined bottom from which the urine runs out and is collected in a suitable vessel.

[1] Published by permission of the Controller, Chemical Warfare Department. This work was done two years ago. It was hoped that an opportunity might arise to clear up some of the points, but as none is likely to appear at present, I have published these notes in the hope that they may be useful.

GOAT CRATE

Length [without extension for trough] .....37½″
Breadth.....13½″
Depth [without trestles].....26½″
Feeding tray.....13½ × 17″
Battens .....3″

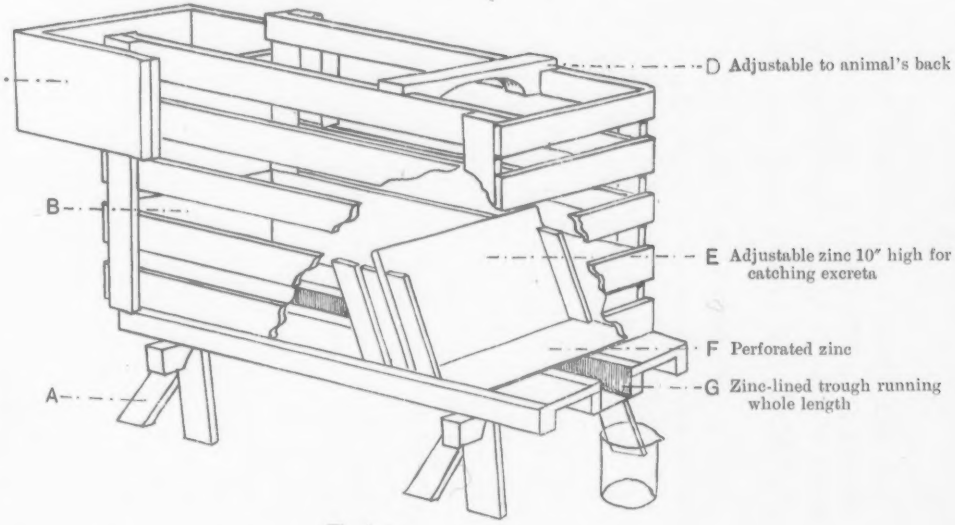
Removable feeding tray C
D Adjustable to animal's back
Zinc tray to top of second batten lining whole compartment
B
E Adjustable zinc 10″ high for catching excreta
F Perforated zinc
Removable trestles
A
G Zinc-lined trough running whole length

Fig. 1.

In order to avoid contamination with faeces billy goats were used. A bag was devised by Lc.-Cpl. Morgan, which is attached to the goat and into which it passes its faeces. This is shown in Fig. 2.

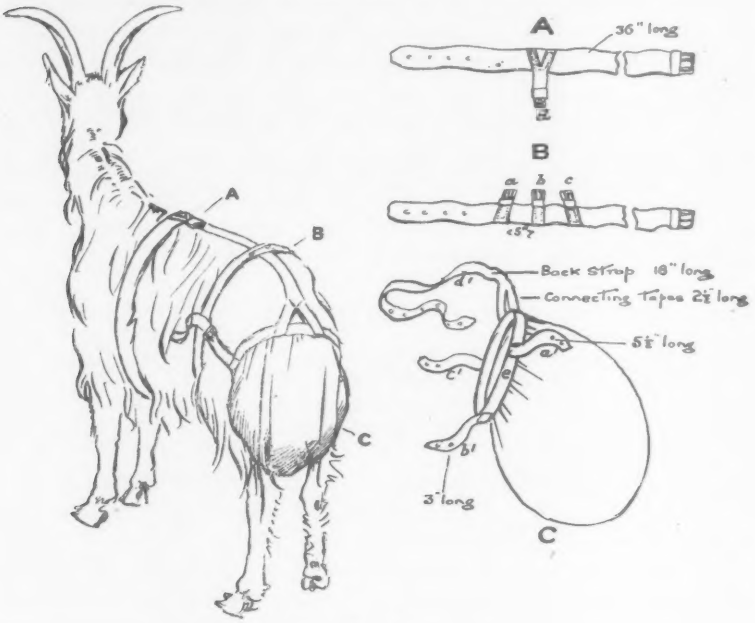

Fig. 2.

A is chest-band made of two-inch webbing, 3 feet in length. *d* in centre buckles to strap *d'* on bag in Fig. 2 *C*.

B is girth-band made of elastic 1 inch in breadth by 2 feet 6 inches in length, *a*, *b*, *c*, in centre fixed on slant, buckled to tapes *a'*, *b'*, *c'* in Fig. 2 *C*.

C is faeces bag of waterproof jaconet; sizes vary with animals. Medium size, flat, length 12 inches, width 8 inches. For a 56 lb. animal width 4 inches is sufficient. A band of elastic (*e*) 12 inches long is fixed round the opening. On this are fixed tapes *a'*, *b'*, *c'*, *d'*, made of elastic to prevent chafing. *a'* and *c'* are 5½ inches, *b'* is 3 inches and *d'* 18 inches in length. *a'*, *b'*, *c'* buckle to *a*, *b*, *c*, in Fig. 2 *B*

To fix.

1. Put on chest-band *A*. If it tends to slip put it between the forelegs.

2. Connect *C* to *B* by the small tape *b'* and buckle *b*. Buckle round the girth-band, and buckle *d'* to *d*; then adjust the other small straps *a'* and *c'*. The back strap *d'* (Fig. 2 *C*) is fastened to girth-band by a large safety pin. The bag is removed easily by undoing the safety pin, and slipping the whole over the beast's legs.

2. *Determination of Hydrogen Ion Concentration.* This has been studied colorimetrically, using a comparometer and indicators similar to those developed by the American workers [see Clark and Lubs, 1917] on the subject. Pheno and cresol red were the indicators used practically throughout. A three-tube comparometer was used similar to that of S. W. Cole.

Standard phosphate, borate and potassium chloride mixtures were used for the comparison.

3. *Titratable Alkalinity.* 10 cc. samples of urine are titrated with acid ($N/10$ HCl) or alkali ($N/10$ NaOH) according as the urine is more acid or more alkaline than $P_H = 7{\cdot}45$. The estimations are made in a comparometer. The control urine tubes receive similar dilutions to the sample that is being studied.

The titration end point has been taken as $P_H = 7{\cdot}45$ which is approximately the reaction of normal blood.

Determinations of titratable alkalinity using phenolphthalein, instead of phenol red or cresol red and boiling off the CO_2 formed, were done on some goats. The results in the main run hand in hand with those obtained by using the $P_H = 7{\cdot}45$ standard, as shown in the following table.

Table I.

Determinations of titratable alkalinity using (1) $P_H = 7{\cdot}45$ as an end point, and (2) $P_H = 9{\cdot}0$ (phenolphthalein) as an end point.

\+ = cc. $N/10$ alkali (NaOH).
− = cc. $N/10$ acid (HCl).

Goat	$P_H = 7{\cdot}45$	$P_H = 9{\cdot}0$ (phenolphthalein)
A.	+ 43·5	+101·5
24 hours sample	+ 85·1	+639·4
	+ 43·5	+375
	+ 25·5	+196·4
	− 21·8	− 29
	−227·5	−214·5
B.	+ 27·0	+ 66·6
24 hours sample	+ 49·5	+253·0
	+ 31·8	+206·5
	+ 33·7	+266·8
C.	− 12·3	− 24·5
	− 5·7	− 37·1
	+ 3·6	+244·0
	+ 15·5	+ 49·6
	− 4·6	− 41·4

The results are parallel on the whole, though there are larger increments in the titration value with phenolphthalein. The comparison was made by Lt. T. W. Wilson, U.S.A.

4. *Estimation of ammonia* (*amino-acid*). Samples have been tested by Sörensen's formaldehyde method with the following modification by Lieut. Wilson for the analysis of alkaline urines. The alkaline urine is acidified to $P_H = 7{\cdot}0$, raised to the boiling point and cooled quickly. After this the estimation is proceeded with in the standard way.

5. *Estimation of chlorides.* The chlorides were estimated by Volhardt's method.

II. Calculation of results and explanation of diagrams.

As a general rule 24 hourly specimens have been collected. The results have been expressed as cc. of $N/10$ alkali per 24 hours, and grams NH_3 per 24 hours.

In a few instances the urine was collected in periods shorter than 24 hours. As the rate of excretion is of most interest here, the results are calculated in times of 24 hour periods, *i.e.* the average hourly rate of excretion during a period over which the specimen is collected is multiplied by 24.

Suppose a 15 hour sample of urine measured 170 cc., the result for the cc. of alkali per 24 hours has been calculated on a volume of 272 cc.

$$(= 170 \times \tfrac{24}{15} \text{ cc.}).$$

In this way the comparison of the rate of excretion in irregular periods is possible.

The results have been plotted on diagrams. In the diagrams the results given for a definite day say 6. viii. 18 represent the amounts excreted, the P_H for the same urine etc., for the 24 hours including the night 6. viii. 18 to 7. viii. 18.

III. The normal urine and its variation with different diets.

As in other animals the acidity and the ammonia excretion in the urine of the goat vary considerably with the diet.

Most of the goats used in these experiments, when taken from the goat pen, excreted an acid urine. They were placed on the experimental clover hay diet. The equilibrium reached by the urine tended to run roughly parallel to the H-ion concentration of an infusion of the hay or grass sample. The infusion was made by heating the hay gently in water and allowing it to stand for two hours.

Good clover hay was alkaline, about $P_H = 8{\cdot}5$. A goat's urine on this diet has also a P_H of about 8·5.

Poorer samples of meadow hay and grass, the latter especially, had a more acid infusion value. Meadow hay infusion had a P_H of 8·0, and fresh grass of between 6·0 and 7·0. The goat's urine on this latter diet always showed a more acid value than on the former. On fresh grass in fact, the urine tended to be actually acid. The time taken to reach this equilibrium was between two and three days. A delay of two or three days would be expected from the fact that fresh food is not received into an empty stomach in the goat, but into a stomach that contains normally a large quantity of food in process of digestion.

The results given in Tables II–IV are typical of those obtained when the normal goat is changed from a diet of grass to one of hay.

Generally speaking, a goat which has been on a grass diet in a pen must have a urine of low hydrogen ion concentration, for at the time of removal from the pen the P_H usually lies between 5·0 and 6·0. In two to three days

on a good clover hay diet the P_H rises and tends to become constant at a value of 8·5. While this P_H remains constant, the titratable alkalinity shows large daily variations.

The excretion of ammonia (and amino-acid) varies from 0·05–0·3 gram ammonia per 24 hours. Amounts larger than 0·6 g. per 24 hours have not so far been found. As would be expected the chlorides show large variations.

Table II. (Goat No. 2403, weight 38 lb.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	P_H	cc. acid 24 hrs
16. iii. 18	22	1¼	—	375	1040	8·4	53
17. iii. 18	24	½	—	265	1040	8·1	26·5
18. iii. 18	24	1¼	—	195	1035	8·5	41
19. iii. 18	24	1¾	—	310	1042	8·5	106
20. iii. 18	24	1½	—	185	1042	8·5	44
21. iii. 18	24	1¼	—	260	1030	8·5	68
22. iii. 18	24	1¼	500	215	1040	8·5	99
23. iii. 18	24	1¼	500	200	1032	8·5	36
24. iii. 18	24	1¼	250	200	1033	8·4	38

No ammonia or chloride estimations.

Table III. (Goat No. 3144, weight 41 lb.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	P_H	cc. acid 24 hrs	NH_3 g. per 24 hrs	NaCl g.	Faeces
16. v. 18	24	1	nil	315	1033	8·5	59·8	0·147	—	F
17. v. 18	24	1⅜	1000	345	1034	8·5	63·8	0·111	—	F
18. v. 18	24	1¼	900	475	1031	8·5	73·6	0·464	—	F
19. v. 18	24	1	500	485	1032	8·5	48·5	0·181	—	F
20. v. 18	24	1½	1500	455	1033	8·5	50·0	0·092	2·13	F
21. v. 18	24	1¼	1050	570	1031	8·5	51·3	0·198	2·66	F

Table IV. (Goat No. 3520, weight 52 lb.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	P_H	cc. acid 24 hrs	NH_3 g. per 24 hrs	NaCl g.
6. vi. 18	24	1¼	2000	520	1022	8·7	72·8	0·0265	1·946
7. vi. 18	24	1⅛	500	395	1036	8·7	98·7	0·026	2·22
8. vi. 18	24	1	1000	230	1040	8·7	37·9	0·027	1·05
9. vi. 18	24	1	500	190	1041	8·7	38·0	0·029	0·111
Goat taken out of the crate with diarrhoea.									
18. vi. 18	24	1	500	205	1042	8·1	8·2	0·070	1·99
19. vi. 18	24	1	1000	235	1043	8·7	44·6	0·05	1·69
20. vi. 18	24	1	1000	230	1040	7·45	nil	0·18	1·99
21. vi. 18	24	1⅛	1000	300	1037	8·1	15·0	0·107	1·20
22. vi. 18	24	1¼	1000	375	1032	8·5	77·9	0·26	1·50
23. vi. 18	24	1¼	1000	325	1040	8·5	52	0·064	2·54
24. vi. 18	24	1¼	1000	300	1042	8·5	66	0·051	3·28

The effect on the urine of a change from rich to poor hay is shown in the case of goat No. 3128 (Table V). From 11. v. 18–17. v. 18 it was fed on a good clover hay diet. The P_H value started low, when it came out of the grass pen, and then rose. It was taken out of the crate from 17–28th. Beginning on 28th it was fed for three days on a poor sample of meadow hay (infusion value $P_H = 6{\cdot}2$), on 31. v. 18 the normal clover hay diet was given to it again, when it reached a proper equilibrium $P_H = 8{\cdot}5$.

Table V. (Goat No. 3128, weight 66 lb.)

Date	Hours	Diet lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	P_H	cc. acid 24 hrs	NH_3 g. per 24 hrs	NaCl g.	
11. v. 18	24	1¼	nil	210	1027	7·2	−4·2	0·117	—	Clover hay diet. Infusion value $P_H = 8{\cdot}5$
12. v. 18	24	1	500	160	1030	6·4	−8·0	0·129	—	
13. v. 18	24	1½	1350	195	1030	8·1	17·5	0·061	—	
14. v. 18	24	1⅔	nil	385	1042	8·5	113·5	0·065	—	
15. v. 18	24	1⅔	875	175	1035	8·1	20·1	0·035	—	
16. v. 18	24	1½	1000	330	1041	8·1	21·4	0·072	—	
17. v. 18	24	1½	1500	280	1040	8·3	49	0·053	—	
28. v. 18	24	2	1000	275	1034	5·0	−50·9	0·225	3·410	Poor meadow hay diet. Infusion value $P_H = 6{\cdot}2$
29. v. 18	24	2½	445	445	1017	5·2	−46·7	0·287	3·09	
30. v. 18	24	1	750	—	—	6·0	(not free from faeces)			
31. v. 18	24	2½	1000	480	1035	8·3	38·4	0·497	6·25	Clover hay diet. Infusion value $P_H = 8{\cdot}5$
1. vi. 18	24	1	1000	950	1023	8·5	195	0·274	3·44	
2. vi. 18	24	1½	500	655	1028	8·5	81·9	0·155	2·60	
3. vi. 18	24	1½	1000	500	1037	8·5	107·5	0·042	1·81	

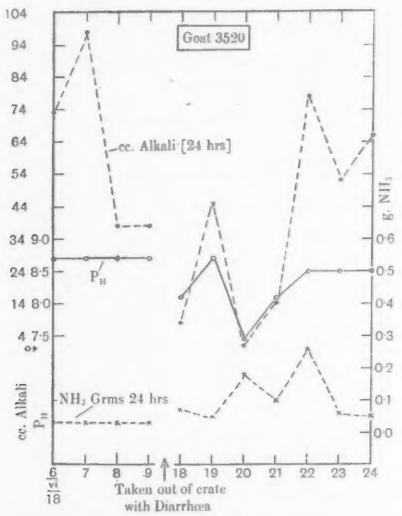

Fig. 3. Showing titratable alkalinity, P_H and ammonia content of urine of Goat No. 3520, normal, fed on clover hay diet.

The case is not quite so simple as it was thought at first, as can be seen from goat No. 4360 (Table VI). This goat was fed throughout on a diet of freshly-cut grass giving an infusion value of $P_H = 5{\cdot}5$–$5{\cdot}7$. For the first three days the P_H dropped, and then rose again, showing some form of compensation. Owing to the urgency of other work further experiments could not be done to settle this point.

Table VI. (Goat No. 4360, weight 58 lb.)

Date	Hours	Diet	Water cc.	Vol. of urine cc.	Sp. Gr.	P_H	cc. acid 24 hrs	Ammonia g. per 24 hrs	
5. viii. 18	24	not weighed	nil	875	1016	8·1	+175·5	—	
6. viii. 18	24	—	500	750	1024	6·7	− 11·2	—	
7. viii. 18	24	—	500	920	1022	6·3	− 18·4	—	Grass diet. Infusion value $P_H = 5{\cdot}5$–$5{\cdot}7$
8. viii. 18	24	—	500	575	1036	7·3	− 2·8	—	
9. viii. 18	24	—	nil	690	1036	8·1	+ 27·6	0·22	
10. viii. 18	No specimen								
11. viii. 18	24	—	500	885	1028	8·1	44·2	0·27	
12. viii. 18	24	—	300	650	1036	8·1	26	0·14	
13. viii. 18	24	—	200	565	1044	8·1	22·6	0·11	

Table VII. (Goat No. 2697, weight 39 lb.)

(The urine was free from protein and sugar.)

Date	Vol. of urine cc.	Sp. Gr.	P_H	cc. $N/10$ acid	Protein	Sugar	Auton
9. iv. 18	245	1028	6·3	+12·3	—	—	—
10. iv. 18	285	1030	7·3	+ 5·7	—	—	—
11. iv. 18*	400	1026	8·4	−36·0	—	—	—
12. iv. 18	310	1026	8·1	−15·5	—	—	—
13. iv. 18	230	1030	7·1	+ 4·6	—	—	—

* Starved 24 hours.

Table VIII. (Goat No. 2720, weight 57 lb.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	P_H	To P_H 7·45 cc. acid 24 hrs	NH_3 g. per 24 hrs	Faeces
9. iv. 18	24	1	—	240	1040	8·4	25·2	—	F
10. iv. 18	24	1½	500	260	1050	8·5	65	0·022	F
11. iv. 18	24	1⅞	500	190	1060	8·1	12·3	0·0014	F
12. iv. 18*	24	nil	500	320	1025	8·3	17	0·032	F
13. iv. 18	24	2	350	270	1034	6·7	−7·81	0·1	F
14. iv. 18	24	1⅞	500	300	1048	8·5	75·5	0·038	C
15. iv. 18	24	1¾	500	210	1050	8·3	21	0·032	F
16. iv. 18	24	1½	1250	1975	1048	8·1	13·8	0·028	F
17. iv. 18	24	2¼	1500	150	1048	7·5	7·5	0·04	F

16th and 17th, traces of albumin.

* Starved 24 hours. F = Urine free of faeces. C = Urine contaminated with faeces.

The data for the urine of goat No. 2720 are shown graphically in Fig. 4.

Effect of Starvation.

Tables VII, VIII and IX show the effect of starvation on the urine of the normal goat. When this period of starvation was 24 hours, the maximum acidity was reached between the 24th and 48th hour after the commencement of starvation; when the period of starvation was 48 hours, the maximal acidity occurred between the 48th and 72nd hour after the last food was taken. In the four cases observed the period of maximal acidity was not during the period of starvation, but after it had ceased in each case.

Table IX. (Goat No. 2879, weight 52 lb.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	P_H	To P_H 7·45 cc. acid 24 hrs	NH_3 g. per 24 hrs	Faeces	Sugar	Albumin
25. iv. 18	24	¾	—	300	1036	6·7	−13·5	·232	—	—	—
26. iv. 18	24	1¼	800	210	1045	6·9	− 6·3	·132	F	—	—
27. iv. 18	24	1	500	195	1048	8·3	+11·7	·071	F	slight trace	—
28. iv. 18	24	1	800	155	1048	8·5	+41·07	·022	F	,,	—
29. iv. 18	24	2	1250	225	1043	8·3	+16·87	·049	F	—	—
30. iv. 18	24	1¾	1250	255	1047	7·8	3·82	·142	F	—	—
1. v. 18	24	1¾	1000	225	1039	8·3	14·62	·070	F	slight trace	—
2. v. 18	24	nil	1250	530	1017	8·3	18·55	·072	—	—	—
3. v. 18	24	nil	125	517	1015	7·6	2·58	·105	F	—	—
4. v. 18	24	1¾	1000	255	1020	6·9	− 6·37	·166	F	slight trace	—
5. v. 18	24	1¼	1250	230	1040	8·3	+13·8	·168	F	,,	—
6. v. 18	24	1	1100	305	1040	8·3	+ 3·81	·067	F	—	—

The Effect of feeding with alkali.

Table X shows the effect of feeding two goats on alkali. In each case 10 g. of sodium bicarbonate was given in a single dose. As in other cases the animal had been kept in the house on a hay diet sufficiently long for the titratable alkalinity of the urine to be constant for a couple of days before the dose. In each case the urine showed a considerable rise in alkalinity on the day of the meal, which had entirely passed off on the day subsequent to it.

Table X. (Goats Nos. 2441, 2453.)

Alkali. Goat No.	Days	Titratable alk.	Remarks
2441	1st	62	
	2nd	110	
	3rd	97	
	4th	169	10 g. $NaHCO_3$ fed
	5th	107	
	6th	27	
2453	1st	5	
	2nd	69	
	3rd	75	
	4th	154	10 g. $NaHCO_3$ fed
	5th	27	
	6th	49	

The course of this experiment on goat No. 2453 is shown in Fig. 5.

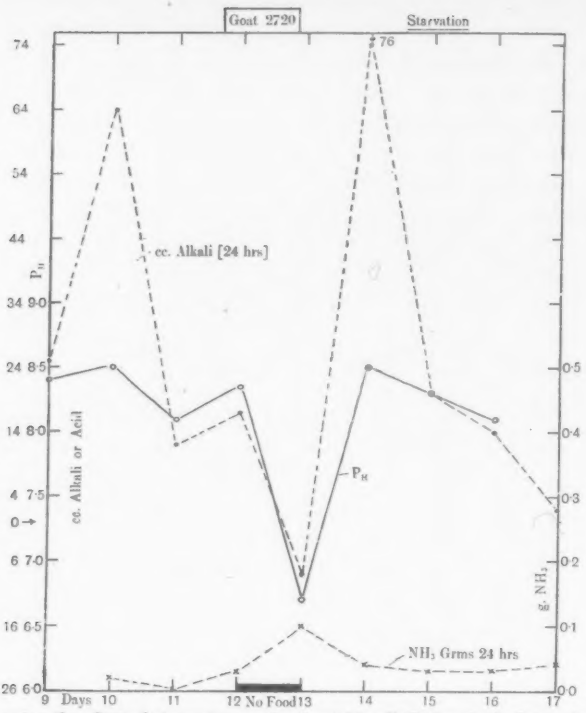

Fig. 4. Showing the effect of starvation on the titratable alkalinity, the hydrogen ion concentration and the ammonia content of the urine of a normal goat (No. 2720, see text).

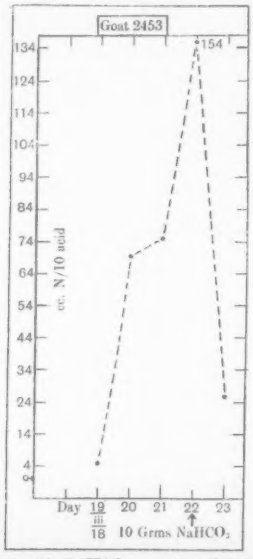

Fig. 5. Effect of feeding with $NaHCO_3$ on titratable alkalinity of goat urine.

IV. Effect of feeding with acid.

Table XI shows the effect of the administration of a dose of hydrochloric acid to a goat. The urine became acid to litmus in the first 24 hour period, the maximum acidity was reached in the period 24–48 hours after administration of the acid. As the urine became acid the excretion of ammonia rose, reaching a maximum in the same period as the maximal acidity and fell as the alkalinity became re-established.

The data which were obtained for the urine of goat No. 2904 are represented in Fig. 6.

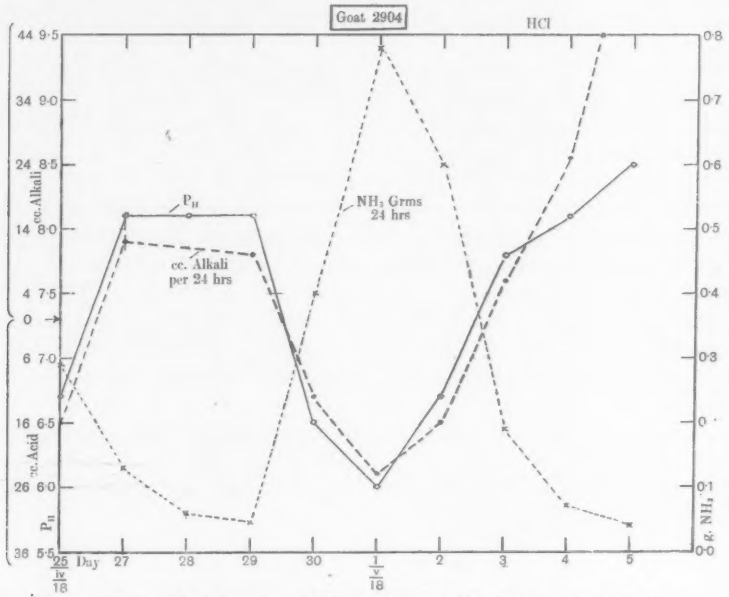

Fig. 6. Effect of administration of HCl on the titratable alkalinity, hydrogen ion concentration and ammonia content of goat's urine.

Table XI. (Goat No. 2904.)

Date	Hours	Diet, hay lb.	Water cc.	Vol. of urine cc.	Sp. Gr.	P_H	cc. $N/10$ acid per 24 hrs	NH_3 g. per 24 hrs	Faeces
26. iv. 18	24	1¼	500	210	1036	6·7	−15·7	0·287	F
27. iv. 18	24	1	250	235	1042	8·1	+11·7	0·125	F
28. iv. 18	24	1	520	220	1038	8·1	14·3	0·057	F
29. iv. 18	24	1½	1000	285	1035	8·1	9·97	0·046	F
30. iv. 18*	24	1	1250	340	1033	6·5	−11·9	0·407	F
1. v. 18	24	1½	1000	375	1033	6·0	−24·3	0·784	F
2. v. 18	24	1½	1500	370	1035	6·7	−16·6	0·600	F
3. v. 18	24	1½	500	305	1033	7·8	+ 6·1	0·194	F
4. v. 18	24	1	1000	330	1040	8·1	24·7	0·072	F
5. v. 18	24	1	1250	335	1037	8·5	68·7	0·039	F

* At 11 a.m. on 30th 30 cc. of strong HCl in 200 cc. water were given by stomach tube.

Discussion.

The use of ammonia by the goat for neutralisation of acid ingested is another case in which the original distinction between the herbivorous and carnivorous animal has broken down. It is in agreement with the work of Steenbock, Nelson and Hart [1914] upon the production of ammonia for neutralisation purposes by the calf. Fitz, Alsberg and Henderson [1907] showed that rabbits after the ingestion of acid excreted an increased amount of phosphates in the urine. Their paper gives no recorded analyses of the ammonia output, and as Winterberg has shown that rabbits fed on oats utilise ammonia for neutralisation, the rabbit would not seem to be really exceptional.

Summary of results.

1. A method is described of obtaining urine specimens uncontaminated by faeces from the billy goat for the study of its metabolism.

2. The results which have been obtained show that the goat's urine responds to alterations in the acidity of the diet. With the addition of acids to the diet, the P_H of the urine gets less, and the titratable alkalinity falls. As the urine passes the neutral point and becomes actually acid there is a tendency for ammonia to be excreted.

3. The goat though herbivorous uses ammonia for the neutralisation of ingested acid, and in this respect confirms the conclusions of others that there is no real distinction between the herbivorous and carnivorous animal in this respect.

4. The goat readily excretes alkali when this is administered by the mouth.

5. The goat's urine reacts to a period of starvation by becoming more acid. In one or two days' starvation the urine reaches its most acid value after the starvation is stopped.

I am indebted to Mr Barcroft for his help, and to Lieut. T. Woodward Wilson, U.S.A. for advice and criticism, and for having performed some of the estimations quoted in the paper. I am also grateful to Lc.-Cpl. Morgan, R.E. for his invaluable assistance.

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LXVI. THE ACTION OF SEA WATER ON COTTON AND OTHER TEXTILE FIBRES.

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The rapid disintegration of textile fabrics when exposed to sea water is well known. Under the condition of complete immersion most textile fibres become completely rotten in three to five weeks. The scientific interest attaching to the decomposition of such resistant materials as cotton and linen by the action of sea water is considerable. It is also of great importance to the marine biologist and others to discover the cause of this destructive action, leading either to a method of protecting fabrics, or to the production of a resistant material. The present paper contains a description of some experiments made with these objects in view.

The general effect of sea water is well illustrated in the following Table I which contains the results of trials carried out in the large tank of the Brighton Aquarium for which I am indebted to Dr J. Lyster Jameson.

Table I. *General Effect of Sea Water on various Textiles.*

+ indicates no obvious sign of weakening. ± visible deterioration. − completely rotten.

	Description	Examined after Three weeks	Five weeks	Ten weeks
1.	Cotton fabric	−	...	...
2.	„ „ treated spermaceti	−	...	...
3.	„ „ treated spermaceti and ironed	+	−	...
4.	„ „ tanned with cutch	−	...	...
5.	Linen fabric	+	−	...
6.	„ „ treated spermaceti and ironed	+	+	−
7.	„ „ tanned with cutch	+	−	...
8.	Worsted fabric	+	+	±
9.	„ „ tanned valex*	+	+	+
10.	Silk fabric	+	−	...
11.	„ „ treated spermaceti	+	+	−
12.	Viscose silk	−	...	...

* This gave way completely in the next four weeks.

The fabrics were mounted in square frames and weighted so as to float vertically with the top edge just awash. After a week nearly all the materials showed a flocculent deposit, apparently bacterial and diatomaceous in

character, which readily shook off, leaving the fabric with a greasy feel which suggested the presence of organisms closely attached to the fibres. Green and rusty brown spots then appeared and gradually the fabrics disintegrated.

As will be seen cotton will not endure an immersion of three weeks, and linen and silk stand less than five. The spermaceti treatment (used by fishermen for preserving their lines) makes very little difference, an interesting result which may be due to the fact that spermaceti, as an ester, is possibly decomposed by micro-organisms. A better mixture would no doubt be paraffin wax combined with a softening agent. The tanning process mentioned consisted of a treatment with 5 % dichromate solution at 60°–65° for one minute. The material was then worked in a 3 % tanning solution (valex or cutch) for one minute and the process repeated several times. The spermaceti treatment was carried out by immersion for 15 minutes in a bath composed of spermaceti two parts, liquid paraffin B.P. 1 part at about 65°.

The tanning of vegetable fibres, however, has little influence, but the author has recently obtained a better result by tanning cotton for one hour at 70° in a 0·05 % tannin solution and then fixing by immersion in solutions of metallic salts the metals of which are toxic to bacteria. With copper a "life" of nine weeks was secured, though antimony and zinc made little difference.

The Chemical Changes produced in Cellulose by the action of Sea Water.

To ascertain the chemical changes produced in cellulose fibres a purified madder bleach calico was used. Test pieces were exposed[1], P in the light, Q in the dark. P soon became covered with pink growths which then disappeared to a considerable extent leaving rusty stains. In three weeks P was falling to pieces while Q, though whole, was quite rotten. P gave the following results:

	%
(*a*) Loss of weight in boiling 1 % NaOH (5 mins)	17·44
(*b*) Loss of weight in boiling 1 % NaOH (60 mins)	17·70
(*c*) Loss of weight in cold 17·5 % NaOH (20 mins)	19·15
(*d*) Hygroscopic moisture	6·09
(*e*) Copper reduced	1·42

The original madder bleach cotton gave under (*a*) 0, under (*b*) 0·6 % loss, (*d*) and (*e*) are normal figures. Of the part soluble under (*c*) only 20 % was reprecipitated by acid.

It is obvious that a very profound change is produced in the cellulose, as 17 % has become soluble in dilute sodium hydroxide solution. The fact that practically nothing more dissolves on further boiling points to the action being superficial and progressive.

[1] These and all subsequent trials were made for me at the Marine Biological Station, Plymouth.

The respective influences of Oxygen, Light and Micro-organisms.

The variables involved in the action of sea water on cellulose are oxygen, light, and bacteria or mould growth. To study their influence a series of laboratory experiments was set up in which all or none of these conditions were present and each was excluded in turn. Pieces of the standard madder bleach calico were placed in large flasks or jars full of sea water obtained from Lowestoft. Suitable devices were used to ensure freedom from oxygen or bacteria, and the immersion was continued in each case for nearly two years except in that of Exp. E, which was removed at the end of one year.

In Table II, which follows, will be found details of these experiments and in Table III the analytical constants of the pieces exposed.

Table II. *Influence of Oxygen, Light and Bacteria: Experimental details.*

Exp.	Weight in grams	Time in months	Exposed to: Oxygen	Light	Bacteria	Notes on Experiments
A	22·68	21	○	○	○	5 litres of sea water in flask, boiled with the cotton air-free and allowed to fill completely from a second flask permanently attached. Added $HgCl_2$ about 1 part in 2000. Conditions well maintained throughout. Cotton yellowish at end.
B	54·66	20	+	+	○	8 litres in covered glass jar. Water kept covered with toluene and oxygenated frequently by blowing purified air.
C	47·00	21	○	+	+	5 litres; oxygen removed by blowing hydrogen for 3 hours. Large bolt-head flask completely filled, with pipette through cork for expansion, etc. Freedom from air well maintained—fabric brownish at end.
D	27·94	22	○	+	○	A duplicate of A; fabric brownish at end.
E	40·46	12	+	○	+	8 litres; large stone jar exposed to the air and oxygenated frequently.
F	35·00	22	+	+	+	5 litres; oxygenated frequently and kept in good light. Growths appeared and disappeared from time to time.

All weights given in this paper are "dry" weights calculated at water-oven temperature. The moisture was always determined on a separate piece of fabric weighed simultaneously.

Table III. *Analytical Constants of the Cotton after immersion.*

Expt.	Exposed to: Oxygen	Light	Bacteria	Relative strength of warp threads	Change in weight %	Ash %	Loss of weight % on boiling with 1 % NaOH: 5 mins.	60 mins.	Copper reduced %	
Original cotton	...	...	...	100*	...	0·13	0·00	0·50	2·4	
A	○	○	○	126	+0·3†	0·21	0·07	0·42	2·4	Least affected— bacteria absent.
B	+	+	○	124	−0·4	0·17	0·16	0·21	1·7	
C	○	+	+	107	−0·6	0·16	0·10	0·65	2·3	Comparatively unaltered—oxygen absent.
D	○	+	○	105	+0·8†	0·12	0·52	0·64	1·9	
E	+	○	+	85	−2·4	0·21	1·80	1·90	2·8	Oxygen and bacteria—most affected.
F	+	+	+	79	−2·0	0·11	0·83	0·97	2·4	

* The actual breaking load was 123 grams.

† Due no doubt to the property of cellulose of fixing mercury from mercuric chloride (Vignon). This was present in Exps. A and D.

These results show clearly that if bacterial growth is prevented sea water as such has no action on cotton. Exps. A and B show practically the same values as the normal cotton, light and oxygen by themselves appearing to be without effect (Exp. B). The curious increase in strength shown by the first four samples is possibly due to shrinkage. On the other hand Exps. E and F, in which micro-organisms and oxygen were present, show marked deterioration comparable in kind, though not in degree, with Exps. P and Q made in the sea. They show a distinct loss in strength and in weight and in addition the remaining cellulose exhibits a solubility of nearly 2 % in sodium hydroxide which is not increased by more prolonged boiling. Light seems to have a slightly accelerating influence on the rate of destruction.

This series of experiments points strongly to the conclusion that bacterial or mould growth is the cause of the destruction of cotton fibres by sea water and that the presence of oxygen is essential.

This conclusion was supported by microscopic evidence. The investigation of a somewhat similar problem, viz. the deterioration of cotton on wet-storage, by Fleming and Thaysen [1920] led to the result that cotton stored damp is not affected unless micro-organisms are present, and not then if the moisture content of the cotton is reduced below 9 %. Finding it impossible to study microscopically the changes in the structure of the fibre brought about by the micro-organisms, these authors applied the modification of the viscose reaction suggested by Balls.

After treatment with the alkali-bisulphide mixture the fibres immersed in water swell to five times their normal size bursting the cuticle if it remains uninjured. In the paper mentioned, photographs are given showing the result of this test.

(*a*) With the normal fibre, presenting the appearance of a series of bulbs connected by rings of cuticle.

(*b*) With the fibres damaged by a streptothrix which has destroyed the cuticle and also perforated the fibre in many places causing it to break up into short pieces (fly).

(*c*) With the fibres damaged by a cellulose-decomposing schizomycete which eats away the cuticle over large areas and causes a progressive destruction from the surface to the interior.

The cotton fabrics E and F which were most affected by exposure to sea water when submitted to similar tests showed of course a large number of normal fibres. A considerable proportion, however, gave evidence of cuticular damage, and in each case the appearance was exactly that assigned by Fleming and Thaysen to the cellulose-decomposing schizomycete. This was abundantly proved by an examination of specimens P and Q (17 % soluble in NaOH). In this case practically every fibre showed evidence of the schizomycete—the streptothrix action was not observed.

The Nature of the Change produced in Cotton by the action of Sea Water.

Some conclusions as to this can be drawn from the figures obtained in these experiments. Numerous trials as with P and Q show that complete disintegration of the cotton takes place. On drying, the piece readily breaks up into a dusty powder. Yet except in the important matter of solubility in alkalies, the material shows very little difference from the normal cellulose. The pieces dyed with methylene blue and benzopurpurin to the same extent as the original fabric. The formation of a reducing substance such as oxycellulose which is almost invariably the result of chemical attack is excluded by the fact that the copper numbers in Table III are identical with the normal value[1]. The action of the micro-organisms would seem to be mechanical and in this connection it is of interest to recall the observations of Cross and Bevan [1918] on the destructive breakdown of cotton fibre under the mechanical process of beetling. A "cellulose dust" is formed which in its proximate composition, copper reducing power, dyeing and mercerising qualities etc., resembles normal cellulose. On treatment with 17·5 % sodium hydroxide in the cold, however, only 44 % remains undissolved and of the 56 % in solution only 48 % is reprecipitated by acids. These curious changes are being critically studied, and the results obtained in the present paper certainly show a similarity which is worth recording. Some marked change takes place in the cellulose complex which results in an entirely new direction of cleavage under the influence of alkali and yet permits of the retention of other characteristic properties.

Hess and Wittelsbach [1920] regard cellulose as a complex built up of "cellulose" (hydrocellulose) molecules united by the residual valencies of the hydroxyl groups. They suggest that the physical characteristic of cotton as a hollow thread is reproduced in the arrangement of the cellulose molecules in the complex, and that this complex can be broken down by mechanical means producing changes similar to those resulting from moderate chemical treatment.

It is exceedingly probable that the change whatever it may be is conditioned by the reactivity of the three hydroxyl groups of the cellulose unit formula and it appears not unlikely that if some or all of these were acetylated or otherwise "protected" the cellulose complex would be unable to rearrange itself in the direction of alkali solubility, and loss of structural character and the deterioration due to beetling or to micro-organisms might not take place. A suggestion on these lines was put forward by C. F. Cross eight years ago and two cotton fabrics were acetylated by his method approximately to the stage of a mono- and a di-acetate respectively, the structural form of the cotton being retained. These pieces were immersed in sea water at the same time as those detailed in Table I. No growths were visible at any time, and when the fabrics were removed after 16 weeks they appeared completely

[1] These numbers though very much higher than usually found for a purified cellulose were done by a standard method and are comparable

unaffected. The process can be carried out by such a method as that of Eng. Pat. 142,615 of Oct. 1919. One part of fused zinc chloride is dissolved in a mixture of acetic anhydride 5 or 6, glacial acetic acid 4 or 5 parts. The cotton is treated several times with double its weight of this mixture at temperatures below 50°. It is very difficult, however, to acetylate an open fabric such as muslin without largely sealing up the openings and the process is not of much use for marine biological purposes.

Cellulose Acetate Silk, a resistant Textile Material.

Attention was therefore directed to the "Acetate Silk" now being manufactured on a large scale by British Cellulose and Chemical Manufacturing Ltd. from the soluble cellulose tri-acetate. Yarns of every grade of fineness are now available and by the courtesy of the Sales Department a supply was sent for experiment.

An acetate silk twist yarn with a breaking load of 3·2 kilos was immersed in sea water under the same conditions as those of Exps. P and Q. At the end of five weeks no growths had appeared, the fibres seemed quite unchanged and the breaking load was still 3·0 kilos. Even after four months no appreciable change had taken place.

It will be seen therefore that a material is at length available which has a satisfactory resistance to sea water. At present fabrics composed wholly of acetate silk are not being manufactured, mixed fabrics only being produced. The yarn is available in any quantity, and it is recommended that this be knitted into suitable meshed fabrics for marine biological use.

SUMMARY.

1. Fabrics of cotton and silk are destroyed by immersion in sea water for three weeks, wool lasting somewhat longer.

2. This destructive action has been shown in the case of cellulose to be due to micro-organisms and not to oxygen, light or the salts present.

3. In its nature it resembles the "mechanical" break-down of cotton sometimes observed under the "beetling" process.

4. If cotton is acetylated to the mono-acetate stage so that its structural qualities are preserved, the resulting material is very resistant to sea water.

5. Cellulose acetate silk now manufactured on a large scale has proved able to withstand the action of sea water for months.

I desire to express my thanks to Dr E. J. Allen, F.R.S. of the Marine Biological Station, Plymouth, for his valuable cooperation throughout this investigation, for the expenses of which I am indebted to a grant from the Dixon Fund of London University.

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LXVII. A NOTE ON THE DIFFERENTIATION OF THE YELLOW PLANT PIGMENTS FROM THE FAT-SOLUBLE VITAMINE.

By MARJORY STEPHENSON, *Beit Memorial Research Fellow.*

From the Biochemical Laboratory, Cambridge.

(*Received October 12th, 1920.*)

The identification of the yellow plant pigments—carotene and xanthophyll—with certain lipochromes of the animal body has been established by Palmer and his co-workers and has formed the substance of an interesting series of investigations.

Palmer and Eckles [1914, 1] first identified the yellow colouring matter of cow's milk with carotene accompanied by insignificant traces of xanthophyll. They showed that cows fed on a carotene-free diet yielded, after some weeks, a completely colourless milk, and further that, on the restoration of green food to the diet, the carotene was immediately diverted to the milk fat. The same writers also showed that carotene—with traces of xanthophyll—forms the yellow colouring matter of the body fat, corpus luteum and skin secretions of the cow and made the further observation that the same pigments form the colouring matter of the serum albumin from which they can be separated by drying with plaster of Paris, and treating the dried mass with alcohol, followed by extraction with light petroleum [Palmer and Eckles, 1914, 2, 3].

On extending these observations to other animals Palmer could discover no trace of carotene in the blood serum of swine on grass feed, traces only were found in the blood serum of the sheep and none could with certainty be isolated from ewe's milk. Goat's milk showed possible faint traces of carotene. Horse serum, on the contrary, showed a content of lipochromes comparable with that of the cow [Palmer, 1916].

Examination of the lipochrome content of the fowl [Palmer, 1915] brought to light interesting differences between the chemical habits of birds and mammals in this respect. It appears that, whilst in mammals the predominant plant pigment stored in the body is carotene, yet in the fowl the pigment stored in the egg-yolk body fat and blood serum is principally xanthophyll. Another striking point of difference is the manner of the association of this pigment in the serum albumin. In the case of the mammal the carotene could not be separated from the blood serum by merely shaking with light petroleum,

in which this pigment is soluble; a preliminary breakdown of the albumin-carotene complex by treatment of the dried mass with alcohol was necessary. In the case of the xanthophyll-albumin complex of the bird mere shaking with light petroleum sufficed to remove the pigment.

The distribution of these yellow plant pigments in animal tissues, and particularly their concentration in the milk of the mammal and the egg yolk of the fowl, has naturally led to considerable speculation as to their function and has tempted several workers to seek for their association or identity with the fat-soluble vitamine. The absence of these pigments from the body fat of the pig—a fat which has been shown to be low in vitamine content—also formed a finger-post pointing in the same direction.

Drummond [1919] fed crude carotene in the proportion of 0·003 % of the total diet and obtained evidence of traces of the fat-soluble vitamine. When, however, pure recrystallised carotene was substituted for the crude product a negative result was obtained. Zilva [1920] found that an alcoholic extract of carrot—which must have contained small quantities of the pigment—carried the fat-soluble factor and when fed in quantities corresponding to 25 g. of fresh carrot per rat per day sufficed to restore normal growth.

Palmer and Kempster [1919] working on the growth and fecundity of fowls obtained interesting results negativing the importance of yellow plant pigments in the animal. These workers experimented first with a ration which they stated to be "carotinoid free"; as however they mention that this ration contained bone meal *ad lib.* it would have been more satisfactory had they furnished evidence that this was fat-free, since from freshly ground beef bones it is possible to extract a bright yellow fat which in view of Palmer's earlier work probably contains carotene. In the course of their investigations, however, they resort to a special carotinoid-free diet in which bone meal was replaced by ground limestone. Hens on this ration laid eggs which were almost but not quite pigmentless; the eggs showed normal fertility, but were very slightly below the normal in percentage hatched. Considerable trouble was taken to prove the nature of the traces of pigment in these eggs. Although this was not actually identified, it was shown that it was neither xanthophyll nor carotene. These experiments showed fairly conclusively that the absence of yellow plant pigments does not affect prejudicially the health or fecundity of hens, and as a consequence renders it highly improbable that the fat-soluble vitamine can be identified with these pigments.

The experiments described in the present paper were begun in June, 1919; while they were in progress, work bearing on the same subject has been published both by Palmer and Kempster [1919] and by Zilva [1920]. The experiments about to be recorded are therefore chiefly of interest as independent corroboration of their results. As, however, our knowledge of the fat-soluble vitamine is at present mainly derived from feeding experiments for which the widest possible statistical evidence is desirable, it has been decided to publish them.

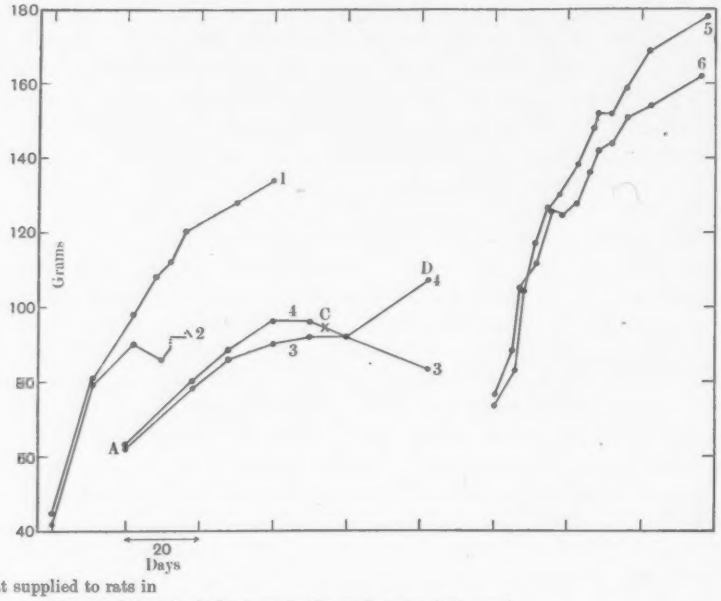
Experiment 1. *The effect of an alcohol-light-petroleum extract of dried*

carrot on the growth of young rats. In order to determine whether carotene is the substance responsible for the growth-promoting properties of butter fat a preliminary trial was made with a crude extract of dried carrot containing the pigment. The fresh carrots were passed through a mincing machine, dried by means of an electric fan between 30° and 35° and the dried substance reduced to powder in a mill. The powder was placed in screw-stoppered bottling jars and covered with 98 % alcohol for 24 hours. The alcohol was then filtered off on a Buchner funnel and the residue covered with light petroleum (B.P. 40°–60°) for 24 hours, again filtered and the light petroleum extraction repeated three times. The yellow alcoholic filtrate was reduced in vacuo to a syrupy residue and the deep red petroleum extracts from the combined washings were concentrated until the volume of the solution was about 0·1 of the original combined extracts. The alcoholic and light petroleum extracts were then mixed, and a portion of the extract corresponding to 250 g. of fresh carrot was evaporated to a syrup on a water-bath and mixed with 250 g. of a palm kernel oil, known by repeated previous experiments to be deficient in the fat-soluble vitamine. Ten young rats (43–52 g. initial weight) were put on the usual synthetic diet employed in this laboratory, consisting of a mixture of starch, sugar, and purified caseinogen to which was added McCollum's salt mixture [McCollum, Simmonds and Pitz, 1916]. The water-soluble vitamine was supplied by a fat-free alcoholic extract of yeast and the anti-scurvy factor by 0·5 cc. lemon juice per rat per day. The fat given was palm kernel oil mixed with the crude extract of carrot as above described. By this means, extract of carrot from 2–3 g. fresh carrot per rat per day was consumed. The growth of these rats is shown on Curve 1. Ten similar rats serving as a control were given the same synthetic diet with palm kernel oil alone. The growth of these rats is shown on Curve 2. After the 36th day the diet of the control set was changed with a view to the study of keratomalacia. No symptoms of eye disease were shown by the rats receiving the extract of carrot, whilst six out of the ten control animals had the disease unmistakably. This, together with the striking dissimilarity in the growth curve, is strong evidence for the presence of the growth promoting factor in the extract of carrot.

EXPERIMENT 2. *The effect of pure carotene on the growth of young rats.* The previous experiment having shown the presence of the fat-soluble vitamine in a crude alcohol-light-petroleum extract of dried carrot, a second experiment was undertaken to determine whether the substance responsible for the activity of the extract was carotene. This was prepared as follows:

5000 g. of fresh carrot were dried as described in Exp. 1, ground and extracted with light petroleum (without preliminary treatment with alcohol) at room temperature; the deep red extract (about eight litres) was concentrated on an electric heater to about 600 cc. and then in vacuo to about 5 cc. The residue was taken up with chloroform (10–20 cc.) and precipitated with methyl alcohol; the crude carotene rapidly crystallised out and was filtered

off under slight pressure. Yield from first crystallisation 0·1678 g. or 0·0033 % of the fresh carrot. The crude product was recrystallised from chloroform and methyl alcohol four times—that is until the M.P. was constant at 172–173° (uncorrected). The final yield was 0·0675 g., *i.e.* 0·0013 % of the fresh carrot, 0·004 g. of the recrystallised carotene corresponding to about 100 g. of fresh carrot was dissolved in 100 g. of palm kernel oil, which thus contained the same concentration of carotene as the palm kernel oil and crude extract of carrot fed in the preceding experiment. Five young rats were fed in the usual way on the standard synthetic diet with palm kernel oil and pure carotene as their source of fat (see Curve 3); five other rats, to serve as a control, were fed



Fat supplied to rats in

Curve 1 (10 rats). Palm kernel oil + crude extract of carrot.
Curve 2 (10 rats). Palm kernel oil.
Curve 3 (5 rats). Palm kernel oil + recrystallized carotene.
Curve 4 (5 rats). A—C. Palm kernel oil.
C—D. Palm kernel oil + crude extract of carrot.
Curve 5 (8 rats). Butter fat decolorised by charcoal.
Curve 6 (8 rats), Untreated butter fat.

with palm kernel oil alone as their fat supply (see Curve 4 A–C). It is seen from these curves that the growth of the rats receiving carotene showed no superiority over that of the control animals and three out of the five suffered from eye disease. The period represented by Curves 3 and 4 covers 82 days. On the 82nd day one rat was killed and the diet of the remaining four

was changed by the substitution of crude extract of carrot for pure carotene; all symptoms of eye disease cleared up in the three rats suffering from keratomalacia. Three of the four, however, failed to survive and died, two on the 14th and one on the 46th day after the change of diet. From this experiment it is clear that the carotene is not the substance responsible for the growth-promoting properties of the crude light petroleum extract of carrot. (On the 54th day of experiment (Curve 4 C) crude extract of carrot was given to the control animals and resulted in the improved growth (Curve 4 C–D).)

EXPERIMENT 3. *The removal of the pigment from butter fat and the effect on its growth-promoting properties.* The decolorisation of butter was carried out as follows: 300 g. of filtered butter fat were dissolved in one litre of light petroleum and about 4 g. of fine birch charcoal added: the mixture was placed in a Winchester quart bottle and shaken for two hours on a shaking machine. At the end of that time the light petroleum extract was filtered free from charcoal and the solution found to be only very slightly tinted; a second treatment with charcoal and subsequent filtration resulted in the complete decolorisation of the butter fat. The light petroleum was then distilled off in vacuo final traces being removed at 60°. The fat thus treated was perfectly white and resembled lard in appearance; it was not ascertained whether this effect was due to the adsorption of the carotene by the charcoal or to oxidation on its surface. It may also be mentioned that only one out of three samples of birch charcoal (all of which bore the same description on the label) had the effect described. An explanation of this anomaly is probably to be found in the work of Philip, Dunnill and Workman [1920] on the activation of wood charcoal by heat.

Two sets of eight young rats were put on experimental diet, the fat in the one case being the decolorised butter fat (see Curve 5), that in the other case being the same sample of butter fat untreated (see Curve 6). One rat on the decolorised butter died on the 14th day of experiment without previous loss of weight and obviously from accidental causes. Perfect growth was maintained in every other individual of both sets for eight weeks after which the experiment was discontinued.

This experiment shows clearly that the removal (or destruction) of the colouring matter of butter does not affect its vitamine content.

SUMMARY.

From these three experiments we may conclude:

1. That a crude alcohol-light-petroleum extract of dried carrot when added to a fat not containing vitamine confers upon it the growth-promoting property and the power of protecting the animal from, or curing it of, keratomalacia.

2. That the substance or substances in the above extract responsible for these properties is not carotene.

3. That the colouring matter of butter fat may be completely removed or destroyed by filtration through charcoal without in the least affecting the vitamine content of the butter.

I wish to take this opportunity of thanking Professor Hopkins for the benefit I have derived from his wide experience in feeding experiments.

The expenses of this Research were in part defrayed by the Planters Margarine Co. and the Maypole Co. to whom my thanks are due.

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LXVIII. NOTE ON THE VITAMINE CONTENT OF MILK.

By FREDERICK GOWLAND HOPKINS.

From the Biochemical Laboratory, Cambridge.

(*Received November 8th, 1920.*)

THE observations described in this note will, it is to be feared, have less interest for others than for myself.

Since, in the early part of 1912, I published a paper describing the startlingly favourable effects of adding minute amounts of milk to synthetic dietaries, incapable, by themselves, of maintaining the growth and health of rats [1912], the conception of Accessory Food Substances or Vitamines has become widely based upon a large mass of experimental evidence. My early experiments have now no more than historic interest.

Since, however, Osborne and Mendel [1920], during their studies of the water-soluble vitamine, have found themselves unable to maintain the growth of rats, when using milk as a source of vitamine in quantities so small as those used by me, I have been naturally anxious to clear up what seemed to be a discrepancy in experience.

I may be allowed the privilege of saying here that the experiments which I described in 1912 followed upon a long experience of the effects of adding tissue extracts, and especially fractionated yeast preparations, to purified diets. Looking back to one's experience in these years during which startling successes were mingled with puzzling failures—failures which led to delay in publication—I realise that the absence of all knowledge concerning the factor associated with fats was the cause of any experimental contradictions. Had I possessed the scientific vision which afterwards led McCollum and Osborne and Mendel to recognise the existence of this, I should have reached full conviction as to the reality of my results long before I did. In the synthetic diets employed by me, the protein and the carbohydrates were purified to the uttermost, but I used little or no discrimination with regard to the fats.

With milk, as an addendum to the purified foodstuffs, I got consistent results in all cases. In preparing at that time a paper for publication I naturally wished to bring out the characteristic quality of the "Accessory Growth Substances" (which in spite of all experimental difficulties had, by 1910–11, become very real to me) of exerting their influence when present in very small amount. I wished in particular to emphasise the fact that their effects had nothing to do with energy supply; hence I used the milk addendum in quantities made as small as possible.

The published experiments, in which quantities of milk as small as 2 cc. per animal per day were employed, were in no sense selected. They were few in number, but gave consistent results and I had no feeling at the time that they would offer any difficulties in repetition.

Some months before they published the paper referred to above, Professors Osborne and Mendel, with great courtesy, communicated the results privately to me.

Even earlier than this I had, as a result of some remarks previously published by Drummond, and by Osborne and Mendel themselves, made, on a small scale, an effort to repeat my original experiments. I became impressed by the apparent influence of seasons on the results.

On the receipt of Osborne and Mendel's private communication, I made a few experiments in the winter of 1919. At this time the results were frankly disappointing. When the animals receiving the 2 cc. of milk in addition to the synthetic diet were compared with others which had the latter alone, the favourable effect upon health and upon the survival periods of the animals was unmistakable. Growth, however, was very slow, and the death rate of the rats was higher than that of animals normally fed.

In April of this year fresh experiments were begun and continued during the summer. The results now became such as to confirm entirely my earlier experiments. Out of 20 animals, each receiving daily 2 cc. of fresh cows' milk as an addition to a highly purified synthetic diet, not one failed to grow almost normally throughout the period of experiment. The observations were not extended beyond 60 days, as they were meant only for comparison with those described in my original paper, and not to determine the amount of milk necessary for continued growth. The technique was exactly that originally used by me. In each experiment control animals were fed upon the synthetic diet alone and the contrast between them and a corresponding set receiving 2 cc. of milk was just as marked as in my original experiments. There has been no selection of results. All the observations made from April onwards are here described.

It may be that such experiments, in which it must be supposed that the supply of vitamines (at any rate of the water-soluble substance) is minimal, are well calculated to bring out the minor variations in the quality of the milk, as it may depend, for instance, upon variations in the summer and winter feeding of cows.

Osborne and Mendel, however, were unable to show that this factor influenced their results. In connection with the experiments just described, I myself endeavoured to get information on the point. Goats were fed upon dietaries corresponding roughly to the winter and summer feed, respectively, of milch cows. Three goats received a mixed cereal diet, together with a small ration of hay and mangold. Three other animals had the same diet, but in addition took grass and green vegetables freely. Two of the animals in each set produced young and their milk was supplied to rats in quantities of 5 cc.

per rat per day. Growth was nearly normal in each case, though, doubtless as an accidental circumstance, the rats upon the "summer" dietary grew rather less well than the others. Analysis of the milk in each case showed that the total solids and fat content was, throughout, almost the same in both. The goats deprived of green stuff produced, however, considerably less milk than the others, a circumstance which would seem to make such comparisons very difficult. It may be when there is deficiency of some essential constituent in the food of a lactating animal that adjustment may occur by a limitation of the total quantity of the milk rather than by a falling off in its quality. The incomplete observation just mentioned, and the experience of Osborne and Mendel, suggest, at any rate, that the apparent seasonal variation in the results of my experiments was not likely to be due to differences in the milk. That there is a seasonal factor in the growth energy of rats is I think sure, but it is doubtful if it could account for the large difference in the experimental results now described. I am endeavouring to obtain further light on the matter. The purpose of this note is merely to point out that given the right conditions my original observations can be repeated.

Experiment 1, begun April 9, 1920. Eight rats were placed upon the synthetic diet. Four received this alone, and four were given 2 cc. of milk each before receiving their daily feed; great care being taken that the milk was completely consumed. The latter grew vigorously during the 60 days of the experiment, the former grew slowly for about 25 days, when their growth ceased and on the 35th day three out of the four died. The individual animals showed a strikingly uniform behaviour in each set. This experiment was supervised by Mrs E. C. Bulley (curves of average growth in Fig. 1).

Experiment 2, begun April, 1920. Of ten rats, five received the synthetic diet alone, and five had the addition of 2 cc. of milk, fed, as before, in advance of the ration. The latter grew normally for 35 days, then somewhat more slowly, but still vigorously, up to the 60th day, when the experiment was stopped. The former grew slowly for rather more than 20 days, after which their weight rapidly fell off, one dying on the 30th day, and the others soon after. For this experiment I have to thank Miss Killby (average curves of growth in Fig. 2).

Experiment 3, begun May 5, 1920. Of 18 rats, eight were upon the synthetic diet alone, and ten received 2 cc. of milk. This experiment, carried out by myself, almost exactly reproduced the results of Experiment 1. The growth curves of individual rats receiving the milk were nearly superimposable, so uniform was their behaviour (average curves in Fig. 3).

Experiment 4. This was the experiment referred to above in which rats were fed upon goats' milk. In Fig. 4 the upper curve shows the average growth of five animals receiving 5 cc. of milk from the "winter" fed goats, while the lower curve refers to five rats receiving the milk of goats supplied with abundant green stuff.

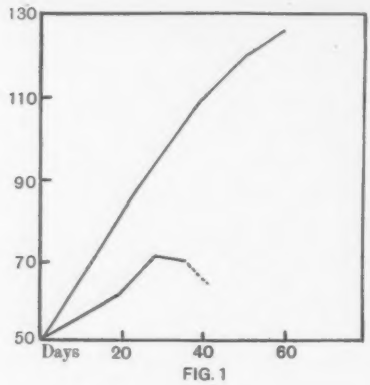

FIG. 1

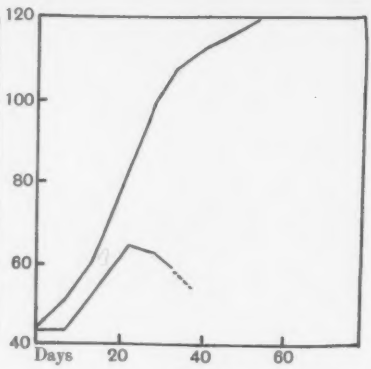

FIG. 2

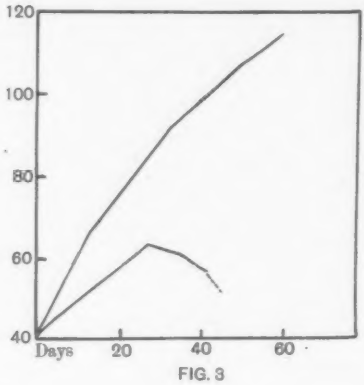

FIG. 3

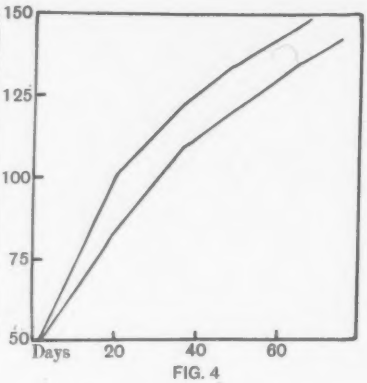

FIG. 4

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LXIX. THE EFFECTS OF HEAT AND AERATION UPON THE FAT-SOLUBLE VITAMINE.

By FREDERICK GOWLAND HOPKINS.

From the Biochemical Laboratory, Cambridge.

(*Received October 15th, 1920.*)

A KNOWLEDGE of the conditions which affect the stability of vitamines is not only of immediate practical importance in connexion with the commercial and domestic treatment of foods, but is, clearly, not without value in setting certain limitations to hypotheses which may be framed as to the nature of these substances, and also in giving guidance when attempts are made to isolate them.

There is at the moment less certain knowledge of this kind in the case of the "fat-soluble A" than in those of the two other recognised vitamines. Thus Steenbock, Boutwell and Kent [1918] came to the conclusion that the substance is readily destroyed by heat, and, later, Drummond [1919] from the results of his earlier experiments came to the same conclusion. Osborne and Mendel [1920], on the other hand, have recently confirmed earlier results of their own which indicated that it is resistant to heat. The investigation to be described in the present paper—to the results of which public reference has already been made [Hopkins, 1920]—confirms Osborne and Mendel's work by showing that the vitamine is relatively resistant to heat. It has demonstrated, on the other hand, that this nutritive factor is rapidly destroyed by exposure to atmospheric oxygen at temperatures ranging from 15° to 120°.

EXPERIMENTAL.

While the main purpose of this paper is to describe experiments which bear upon the relative importance of temperature and aeration as factors concerned in the destruction of the vitamine A in butter, it will be useful to refer first very briefly to an investigation undertaken with a somewhat different aim.

I. *Is there any Destruction of Vitamine during the Commercial Purification of Vegetable Fats?*

When vegetable fats are prepared for use in foods such as margarine they are of course first purified. It is a point not without importance to decide whether the processes applied have or have not destroyed vitamine which may have been present, to some extent at least, in the original crude fats.

Some two years ago I was asked by the firms mentioned at the end of this paper to investigate this question. The fats employed were palm kernel oil and ground nut oil, each respectively in the crude condition and purified for use. A large number of rats was used in testing the point, but it is not proposed to describe the results in detail. The practical issue was that in the case of neither fat could any difference in vitamine content be found between the crude and purified material. The average growth curves of the animals fed respectively upon the crude and pure fat were almost super-imposable, and the average curves of those upon the palm kernel oil were exactly similar to those upon ground nut oil. The results indeed may be grouped together as a whole to yield statistics regarding the behaviour of the rat-stocks in my laboratory when on a diet free from—or greatly deficient in—the fat-soluble vitamine. Except for the fats the diet was identical with that employed in all the experiments mentioned in this paper. In the case of 60 of the animals used, coming from the same or strictly comparable stocks, the average date upon which growth ceased was the 25th day, and in respect of this datum individual animals were closely grouped round the mean. The date of death varied of course much more widely. On the average it was the 56th day. 7 % were dead on or about the 30th day; 12 % died between the 40th and the 50th, 54 % between the 50th and 60th, and 27 % outlived the 60th day. These data, since they were obtained from rat-stocks which were essentially the same as those used for the experiments described in the next section, are of value for comparison.

II. *The Effect of Heat and Aeration on the Vitamine Content of Butter.*

In all the experiments carried out in this connexion the diet comprised highly purified caseinogen, potato starch, cane sugar, butter. The mineral supply was Osborne and Mendel's salt mixture with the addition of 0·10 % potassium iodide and a few milligrams per cent. of sodium fluoride. The butter was always first filtered and, however subsequently treated, formed always 15 % of the food.

The number of animals employed in the whole investigation was large. The growth curves if given in every case would occupy much space, so that certain of the observations must be dismissed with a verbal description. Most of the experiments were made comparative; balanced sets of animals being fed side by side and the one significant factor alone varied in their respective diets. Many authors seem content to compare growth obtained upon a given experimental dietary with the accepted "curve of normal growth" and not with a curve obtained at the time of the experiment from comparable animals on a normal dietary. It is the experience of my laboratory that the curve of normal growth varies both with the season and with the stock. Osborne and Mendel have, I think, commented upon the influence of season on their experimental results. With my stock we seem always to obtain the clearest and most satisfactory results in respect of growth curves during the periods from the

beginning of April to the end of September. This is not a matter of temperature as the laboratory is always uniformly warmed. In the present investigation the comparison has frequently been, not between animals upon the experimental dietary and others upon normal food, but between one set upon fat treated in a given fashion, and another set upon the fat treated differently. Such sets have always been fed side by side.

There is doubtless even greater rigor of proof in the technique used by some others which consists in attempting to restore animals which are presumed to have failed owing to the absence of this or that constituent from their diet by a subsequent supply of that constituent. This method we have occasionally used in the present investigation as in Exp. 6. There are some advantages in the comparative method however.

Experiments 1 *and* 2. Early in 1919 eight rats averaging 57 g. in weight were placed upon a dietary in which the fat was butter previously heated in a steam autoclave for four hours at 120°. Eight similar rats had the same food but with the butter heated at 120° in an oil bath and simultaneously aerated for 12 hours with a brisk stream of air. The fat entirely lost its yellow colour. In the former set, three of the animals exhibited somewhat slow growth from the first, and died before the 60th day, but the remaining five grew vigorously and remained in good health for 160 days when the experiment was stopped. In the case of the second set all the animals failed to grow after the 21st day, five deaths had occurred by the 50th day, while the survivors afterwards developed keratomalacia and were killed. At about the same time eight other rats received butter heated for 12 hours to 80°, in such a way that a considerable surface of the fat was exposed to air. Growth was slow from the first; four deaths occurred about the 60th day, and a fifth occurred shortly afterwards. The three survivors lived to the 140th day when the experiment was stopped; but they had long ceased to grow, and were in poor condition.

These preliminary experiments were sufficient to offer a strong suggestion that aeration is an important factor in the destruction of the vitamine, but the comparison was faulty in the case of the first two sets of animals, since the aerated fat was heated thrice as long as the unaerated.

Experiment 3. In this case the comparison was rigorous, but the experiment was begun when but few young rats happened to be available for the purpose, so there were only four animals in each of the sets compared. The butter was in the one case autoclaved at 120° for four hours, and in the other heated to the same temperature and period but at the same time thoroughly aerated. The first set grew vigorously and remained in perfect health; the set on the aerated fat ceased to grow about the 25th day, and all died about the 50th day.

Experiment 4. This was a crucial experiment. Two carefully balanced sets of rats were taken, with ten animals in each set. One set received the standard diet with butter previously heated to 120° in the autoclave for four hours; the other set had the same diet with the butter heated at the same temperature

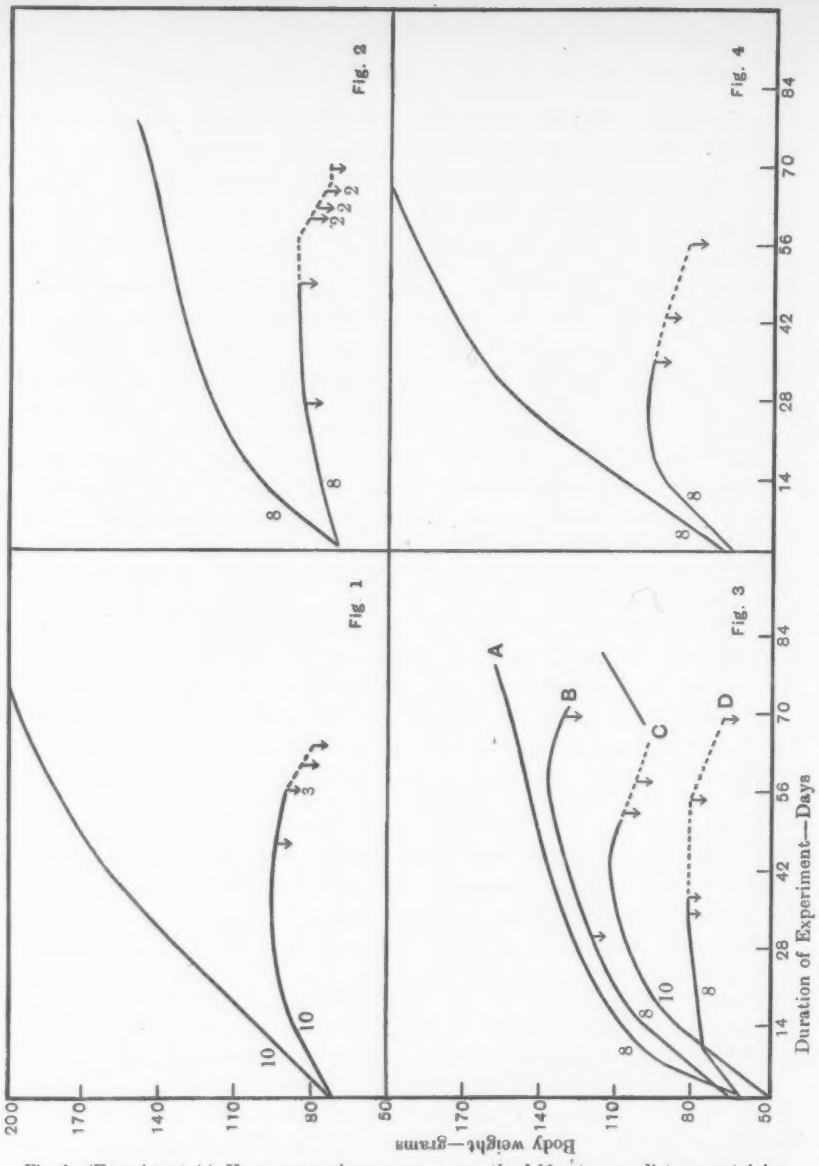

Fig. 1. (Experiment 4.) Upper curve shows average growth of 10 rats on a dietary containing butter heated out of contact with air for 4 hours at 120°. Lower curve shows growth of 10 comparable animals depending upon butter heated for the same time and at the same temperature but simultaneously aerated.

Fig. 2. (Experiment 5.) Curves as in Fig. 1, but the animals were fed with butter respectively autoclaved and aerated for 12 hours at 120°.

Fig. 3. (Experiments 6, 7.) Curve A, animals fed with butter aerated for 1 hour at 120°; B, aeration 2 hours; C, aeration 4 hours; D, aeration 16 hours. The unbroken line continuing Curve C shows the growth of surviving rats when put upon fresh butter.

Fig. 4. (Experiment 8.) Lower curve: animals depending on butter exposed in thin layers to air at ordinary temperatures. Upper curve: animals on butter preserved out of contact with air.

The arrows indicate deaths. When a death appreciably affected the average weight of the group the continuity of the curve is maintained by calculating the weight changes of the survivors on a percentage basis. Change in the curve from a continuous to a dotted line indicates that this has been done. The figures adjacent to the curves indicate the number of animals comprised in each set; those below the arrow give the number which died on a particular date.

and for the same time but with simultaneous aeration. The aeration as in other experiments was carried out in a flask immersed in an oil-bath. By means of a filter pump a stream of air was drawn through the melted fat at the rate of four or five bubbles per second.

The average growth curves obtained in this experiment are shown in Fig. 1, and in Fig. 5 are shown the curves of individual growth. In the latter the curve of each animal in one set is reproduced side by side with that of its representative in the other set, each being of the same sex and so far as possible of the same initial weight. (Two extra rats were in the set receiving aerated fat, for which, when the experiment was started, no exact duplicates could be found. These appear in the curves of individual growth, but they did not contribute to the average curve.)

None of the animals upon the autoclaved butter displayed keratomalacia during the course of the experiment. Of those receiving the aerated butter, three showed symptoms of this disorder on the 40th day, three on the 42nd, three on the 53rd, and one on the 64th day.

Experiment 5. The conditions in this experiment were exactly those of No. 4, but the butter was respectively autoclaved and aerated for 12 hours instead of 4 (120°). Curves of average growth in Fig. 2. To save expense of reproduction individual growth curves are omitted in the case of this and subsequent experiments. The degree of variation seen in Exp. 4 (Fig. 5) is quite typical of other experiments. There was no keratomalacia in the case of the animals receiving the fat heated out of contact with air. Of those on the aerated butter all developed keratomalacia between the 40th and 43rd day.

Experiment 6. Rats belonging to the same stock were divided into three groups with eight animals in each, the groups being balanced in respect of sex and weight. The diet was the same as in all other experiments, but with the butter aerated at 120° for one, two and four hours respectively. A fourth set of animals comparable with the others in respect of sex and weight but from a different stock, and fed at an earlier period, took the diet with butter aerated at the above temperature for 16 hours. The resultant growth curves are shown in Fig. 3.

There was no keratomalacia among the animals receiving fat aerated for one or for two hours; but, in marked contrast with the animals of Exp. 4, there was also none when the butter had received four hours' aeration. The rats concerned came from a stock quite distinct from that employed in Exps. 4 and 5. In the case of the group upon the fat aerated for 16 hours eye trouble developed in several of the animals, but no accurate record of its incidence was kept in this case.

Experiment 7. Filtered butter was kept at 80° and aerated with a brisk stream of air for 12 hours. Eight rats received the usual dietary with this aerated butter as its fat constituent.

The average growth curve corresponded so exactly with that of the animals

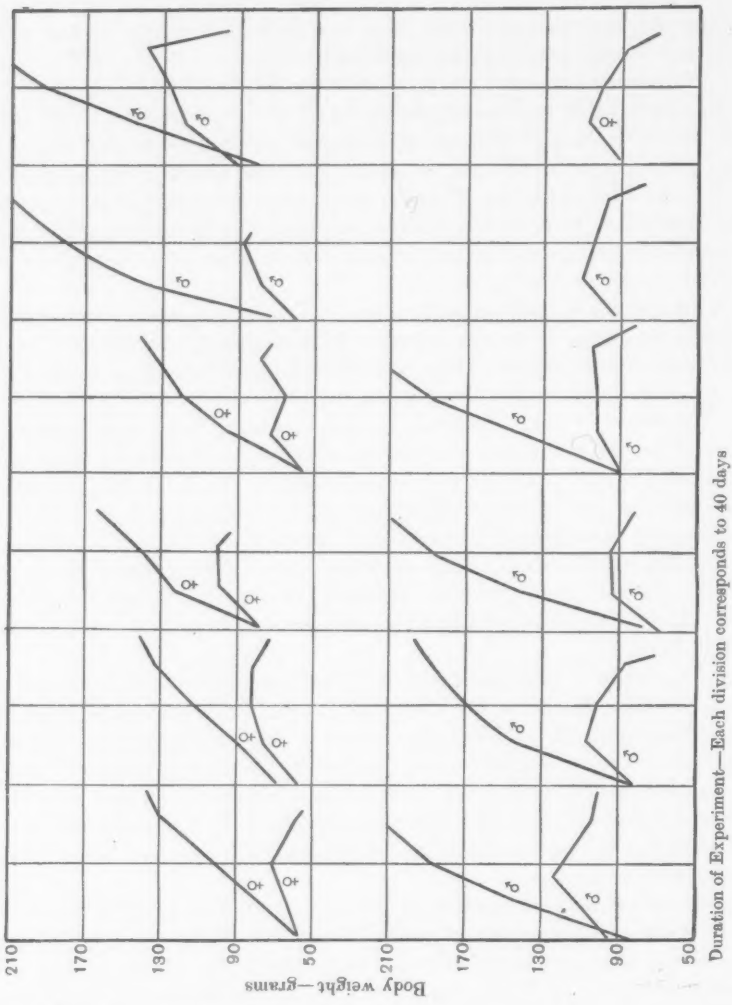

Fig. 5. Growth diagrams of individual rats in Experiment 4.

The upper line in each case shows the changes in body weight of an animal from the group receiving butter heated but not aerated. The lower line refers to an animal corresponding to the first in sex and initial weight, but from the group which depended upon aerated butter. Two rats in the second group were not represented in the first.

In every case the end of the lower curve marks the death of the rat.

which in Exp. 6 received butter aerated at 120° for four hours (Fig. 3, Curve C) that it need not be reproduced. Keratomalacia occurred in four cases between the 40th and 60th days.

Experiment 8. Filtered butter was exposed in thin layers at ordinary room temperatures, varying between 15° and 25° (during May, June and July) for periods of about a week. It had by this time lost all, or nearly all, its pigment. The fat so treated was added to the standard diet in the same proportion as in all other experiments, and the animals receiving it were compared with another set upon butter kept *en masse* for corresponding periods without exposure to air or light. The average growth curves (from eight rats in each set) are shown in Fig. 4. The animals were of the stock used for Exp. 6, different therefore from those of Exps. 4 and 5. It is noteworthy that though growth and nutrition failed on the exposed fat keratomalacia developed in one rat only and not before the 65th day.

Discussion of Results.

It will be seen that the experiments described show in the clearest way that while the fat-soluble A factor is relatively resistant to the effects of heat alone, it is easily destroyed by aeration, presumably because it is a substance prone to oxidation.

It was on these points, and on these alone, that information was sought when the experiments were first planned, and for such a purpose the comparative method used seemed to be the best. The animals, in balanced sets, were fed side by side, aeration or non-aeration of their fat supply being the only difference in the treatment of the animals in the sets so compared. The difference in their behaviour was always unequivocal and most striking.

My experiments, however, give no quantitative information as to the *rate* of the destruction of the vitamine, whether by heat alone or with combined aeration at a given temperature. For this extended experiments and a different technique are called for.

The observations indicate that four hours' exposure to 120° does not, in the absence of air, sufficiently reduce the vitamine content of butter to make the heated product any less efficient than normal butter for maintaining rats, at any rate when the fat forms 15 % of their food (Exp. 4). Twelve hours' exposure to the same temperature seems undoubtedly to involve some destruction, but in the absence of air the vitamine is far from being completely destroyed (Exp. 5). On the other hand, aeration for four hours at 120° destroys the greater part of the vitamine, and exposure for 12 hours to the same condition involves what would seem to be almost complete destruction (Exps. 4, 5, 6). At the same temperature, one hour's aeration renders butter definitely less efficient for maintenance than unheated butter, and an increase in the destruction after two hours could be demonstrated (Exp. 6).

Since heating alone for four hours at 120° produced no effect which could

be demonstrated by the method of feeding adopted (*i.e.* with 15 % of fat in the dietary) and exposure to this temperature for even 12 hours produced relatively little, I have not troubled to test the effect of lower temperatures without aeration. Proof that fractions of the vitamine have been destroyed calls of course for experiments in which the total fat administered is reduced to the critical minimum.

With simultaneous aeration destruction of the vitamine proceeds with considerable rapidity at 80° (Exps. 2 and 7).

Very noteworthy are the results of full exposure to air at ordinary room temperatures. Butter exposed in thin layers at 15°–25° for periods of about a week was found to have lost its power of maintaining the growth or health of rats (Exp. 8). It is possible that in this experiment insufficient attention was paid to the accessory influence of light.

Aeration as is well known bleaches butter, but it now seems fully proved that the carotinoid pigments are not related to the fat-soluble A substance.

Though the hypothesis seems to me *prima facie* unlikely it may occur to some that combined heating and aeration might produce toxic products, or deleterious changes in the fat itself. In this connexion it is not without interest to point out that the general behaviour of the rats depending upon butter aerated at 120° for four hours or longer was very similar to that of the animals referred to earlier in this paper which depended on vegetable fats. In the latter growth continued on the average for 25 days and the average (from 60 rats) duration of life was 56 days. In the former the average period which elapsed before growth ceased was 23 days, and the average (from 42 rats) duration of life was 54 days. Aerated fat has frequently been added to the food of animals upon ordinary diet without deleterious effects.

With regard to the effect of the aeration upon the butter fats themselves, I may point out that Miss M. Stephenson has made determination of the iodine value of the fatty acids of butter before and after heating for four hours in a stream of air at 120°. The figures were identical (*e.g.* 39·0 before and 39·0 after).

Keratomalacia was for some reason more frequent in the animals upon aerated butter as just described than in those upon vegetable fats in the earlier investigation mentioned above. The latter formed part of the material used by Miss Stephenson and Miss Clark for their recently published [1920] study of the disorder. They found that only 28 % of the animals were affected. Taking all my animals upon butter aerated at 120° for four hours or longer one finds that just over 60 % showed symptoms of eye trouble. But although the complete absence of keratomalacia from the rats given heated, but unaerated, butter and its frequency upon the aerated fat is of interest as giving further proof that the loss of nutritive value from the latter is actually due to destruction of the fat-soluble A, I do not attach statistical importance to the above figure. The influence of stock, and perhaps of the nutrition preceding experiment, may affect the incidence of keratomalacia. In Exp. 4 (fat aerated

four hours), and in Exp. 5 (aeration 12 hours), all the animals ultimately developed the condition. In Exp. 6, on the other hand, the animals upon butter aerated for four hours all remained free from it. The last were from a wholly different stock.

Summary.

Experiments carried out upon a large number of rats have shown that the fat-soluble A substance in butter, while displaying marked resistance to heat alone at temperatures up to 120°, is readily destroyed by simultaneous aeration of the fat, presumably because it is a substance prone to oxidation by atmospheric oxygen.

The expenses of this research were defrayed from funds supplied by the Planters Margarine Co. and the Maypole Co.

To Mrs E. C. Bulley I am indebted for valuable help in the general supervision of the experiments.

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LXX. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY FACTOR (VITAMIN A). VI: EFFECT OF HEAT AND OXYGEN ON THE NUTRITIVE VALUE OF BUTTER.

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(*Received October 18th, 1920.*)

A number of somewhat conflicting statements regarding the influence of heat on the vitamin A are to be found in recent papers. Originally this substance was considered to be comparatively stable to high temperatures, for Osborne and Mendel [1915] had found that butter fat lost little or none of its growth-promoting properties when subjected to the action of live steam for two and one-half hours.

Later, however, experiments were recorded by Steenbock, Boutwell and Kent [1918], the results of which cast some doubt on the supposed stability of the vitamin. They found, for example, that butter aerated for 12 hours at a temperature of 100° completely lost its power to maintain growth. Naturally their first tendency was to ascribe the destruction to oxidation, but when they found that shaking melted butter fat with carbonated water for several hours caused inactivation of the vitamin just as readily as when the shaking was carried out in the presence of air, they were led to conclude that the heat alone was responsible.

A series of experiments on the effect of heat on the vitamin present in certain oils was carried out by Drummond [1919], who obtained results which appeared to support the conclusions of Steenbock and his collaborators. In these experiments reliance was placed upon observations of the iodine value in order to ascertain whether the destruction of the vitamin is a process of oxidation. The results indicated that oxidation was not the cause of destruction of this dietary factor at high temperatures. Early in 1920 a paper appeared by Osborne and Mendel [1920], in which they produced further evidence in favour of their view that the vitamin present in butter is stable to relatively high temperatures. They not only confirmed their earlier result on the treatment of butter fat with steam, but also found that a sample of butter fat heated to 96° for 15 hours retained its growth-promoting activity.

In view of their results it was obvious that the question required re-investigating, and accordingly we carried out a new series of experiments in order to seek the cause of the discrepancies.

The result of this work has been to establish that destruction of the growth factor A does take place with ease at high temperature, provided facilities for oxidation are present.

While these experiments were in progress we learnt from Professor Hopkins that he had also arrived at a similar conclusion [1920, 1, 2], and further confirmation is given by the experiments of Zilva [1920]. It is particularly interesting to note that Hopkins has observed that destruction of the vitamin at high temperatures may occur without an appreciable change in the iodine value of the accompanying oil.

Experimental.

Throughout these experiments we employed the technique described in a previous paper [Drummond and Coward, 1920]. The various samples of butter were tested by administering them to young rats whose growth had been completely inhibited by withholding the factor A from their diet. The daily ration of butter was always given separately to the animals before they received the day's ration of the basal diet. In order to be able to detect any destruction of the vitamin, the amount of the daily supplement was fixed at 0·2 g., a quantity which, in the case of the sample of butter used in these tests, was found to give slow but steady growth.

Effect of Exposure of Butter to High Temperatures.

I. *Experiments at* 100°. A sample of the butter was subjected to the action of a current of live steam for six hours, an exposure longer by three and a half hours than that employed by Osborne and Mendel. Experiments on rats showed that this treatment had caused practically no loss of the growth-promoting factor A, an observation which is in agreement with that of the American workers. This treatment would of course effect the removal of the air in the vessel by the current of steam (Curves 1–3, Fig. 1).

II. *Experiments at* 96°. Quantities of butter were heated to 96° for 15 hours, as was done by Osborne and Mendel [1920]. One sample was placed in a large shallow dish in order to expose a considerable surface to the air, whilst another was enclosed during the heating in an air-free vessel. As may be seen from Fig. 1 destruction of the growth-promoting properties took place in the sample exposed to air (Curves 4–6) but not in that protected from oxidation (Curves 7–9).

Further experiments in which two samples of butter were heated to 96° for only three hours with and without air, respectively, gave similar results (Curves 10–12 and 13–15, Fig. 1).

III. *Experiments at* 50°. In order to re-investigate the effect of heating at comparatively low temperatures, another series of butter samples was heated for six hours at 50°. The results as a whole support the conclusion that oxidative processes are responsible for destruction of the vitamin A. As will

be seen from the Curves 16–18, Fig. 2, one animal (16) fed on the fat heated out of contact with air grew as if no deterioration in the nutritive value of the butter had occurred, but two others (17, 18) showed the opposite result. On the other hand, the animals fed on the exposed fat showed that this had been inactivated (Curves 19–21, Fig. 2).

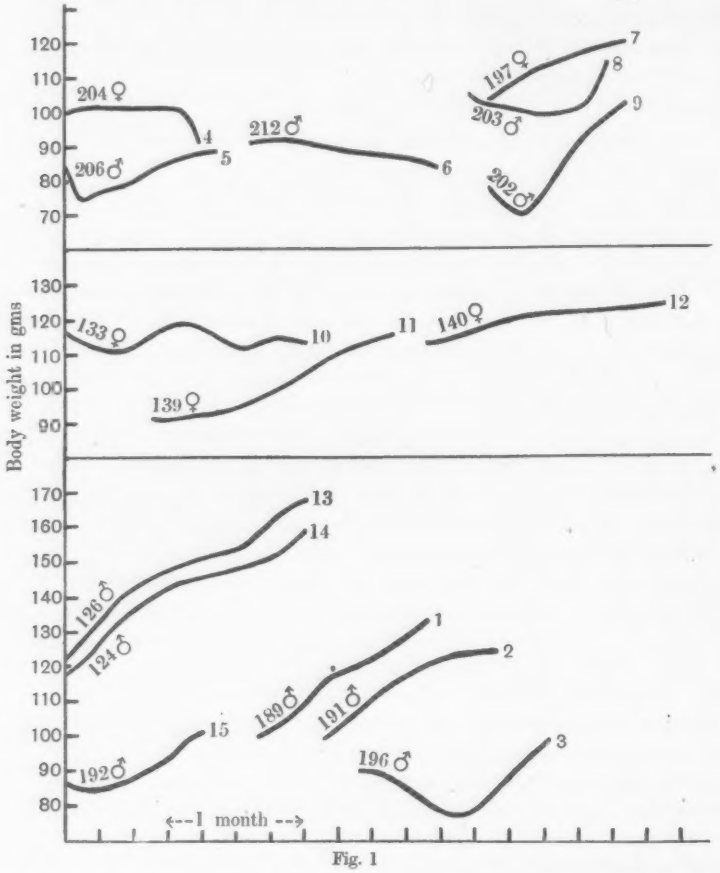

Fig. 1

N.B. *In all the curves presented in this paper the preliminary period when the rats were fed on the basal diet alone is omitted.*

A third sample of butter which was exposed to air in a vessel permitting a much smaller surface to come in contact with the atmosphere was only slightly inactivated (Curves 22–24, Fig. 2).

IV. *Experiments at* 37°. The most striking observation recorded in the earlier investigation [Drummond, 1919] was the instability of the vitamin A

at temperatures as low as 37°. It seemed, however, advisable to repeat the experiments employing the more satisfactory method of testing fractions which we now use. Four samples of the butter were heated at a temperature of 37° for a period of three weeks. Of these, two were exposed to air in thin layers in shallow glass dishes, whilst the other two were protected from air in closely stoppered vessels.

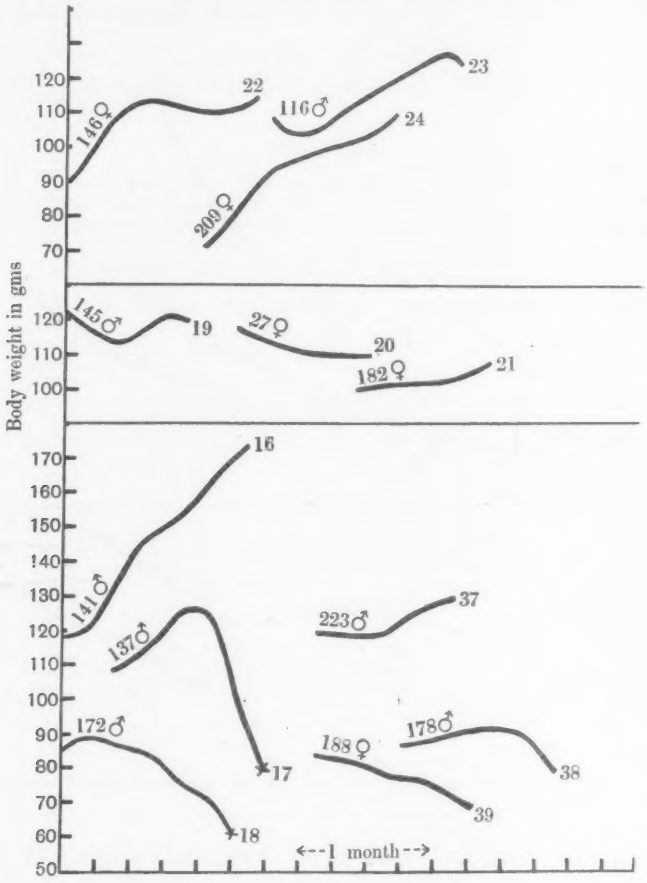

Fig. 2

One of each group was kept in a light corner of the laboratory, whilst the other two were placed in a darkened chamber. By this means it was hoped to ascertain the influence of light and air, since it has already been shown by Zilva [1919] that the vitamin A is destroyed on exposure to ultra-violet rays, a process which he suggested might be due to the action of the ozone

produced by the rays. Zilva has now obtained evidence that the cause of inactivation is oxidation by ozone [Zilva, 1920]. The samples exposed to air in this laboratory both became bleached, that kept in the light almost completely, and they were found at the end of three weeks to have lost their power of restoring growth in rats (Curves 25–27 and 32–34, Fig. 3). No appreciable impairment of the nutritive value of the samples protected from air was detected (Curves 28–30, and 31, 35, 36, Fig. 3).

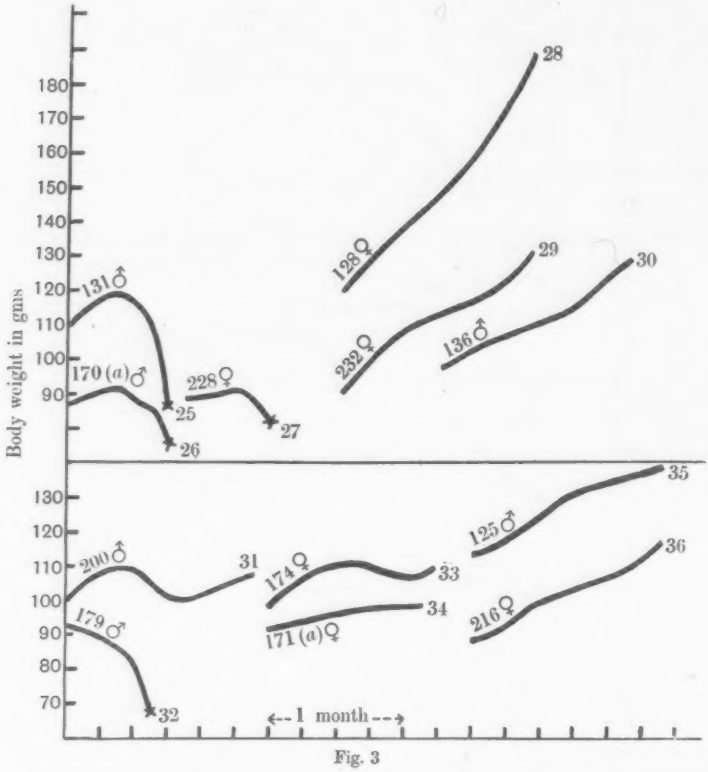

Fig. 3

In connection with these experiments we also tested a sample of butter fat, which Dr O. Rosenheim of King's College, London, very kindly placed at our disposal. This sample had during a previous investigation in 1917 been found to possess considerable growth-promoting power. It had been then dissolved in alcohol, and the solution allowed to stand for many months in the laboratory. When he sent it to us all the alcohol had evaporated, and there remained behind a perfectly white fat resembling a soft tallow. Tests on rats showed that the vitamin had been destroyed (Curves 37–39, Fig. 2).

SUMMARY.

1. Destruction of the vitamin present in butter fat occurs on heating in the presence of air. It is therefore probable that the loss is due to changes of an oxidative nature.

2. The destruction takes place rapidly at high temperatures, but provided the exposure to air is extensive, considerable loss of nutritive value may take place at temperatures as low as 37°.

The authors wish to express their indebtedness to the Medical Research Council for a grant out of which the expenses of this research were defrayed.

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LXXI. THE ACTION OF OZONE ON THE FAT-SOLUBLE FACTOR IN FATS.

PRELIMINARY NOTE.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(*Received October 18th, 1920.*)

In a previous communication [Zilva, 1919] it was shown that when butter was spread in a thin layer on a plate and exposed to ultra-violet rays for six to eight hours, the fat-soluble factor in it was inactivated. It was then pointed out that as ozone was produced by the mercury quartz lamp from the atmospheric oxygen the butter was at the same time exposed to the action of this gas, and that further investigation would be required in order to ascertain whether the inactivation was due to the action of the rays or to that of the ozone. It was therefore decided to establish the influence of ultra-violet rays on the fat-soluble factor in the absence of oxygen and the action of ozone on it in the dark, as well as to determine the iodine value of the fat under different conditions of exposure. As the results were of great interest, the inquiry into the action of ozone was extended to the antineuritic and antiscorbutic factors. The object of this note is to give a brief summary of the results obtained by studying the action of ozone on the fat-soluble factor only. The experimental data are reserved for another communication in which the influence of ozone on all the accessory factors will be described and discussed in detail.

For technical convenience active fats which are transparent and liquid at room temperature, like whale oil and cod liver oil, were chosen for this investigation. The latter oil being very active was found to be the more suitable and most of the experiments were therefore carried out with it. On exposure for six to eight hours in shallow layers in Petri dishes to the action of the ultra-violet rays in an atmosphere containing ozone these oils were entirely inactivated.

Unlike the butter, however, the cod liver oil was not bleached but assumed a slightly darker colour, which suggests that the nature at least of some of the colouring matter associated with it is different from that of butter.

In order to study the effect of ultra-violet rays on the fat-soluble factor in cod liver oil in the absence of oxygen the oil was placed in a thin layer a few millimetres thick between two tubes, one of which fitted loosely into the

other. The outer tube was made of quartz, so that the ultra-violet rays could reach the oil without being previously absorbed. The air above the layer of oil was displaced by carbon dioxide gas, and the tube was revolved by a water motor during the exposure. On testing the exposed oil on rats receiving a diet deficient in the fat-soluble factor it was found that an exposure even of 16 hours' duration did not inactivate the oil, nor was there any evidence that the activity of the oil was impaired to any great extent by such an exposure.

The next set of experiments was carried out with cod liver oil exposed to ozone in the dark. This was done by introducing some of the oil into a dark-stained glass bottle through which a current of ozone was passed. By rolling the bottle at short intervals the oil was thoroughly exposed. About ten hours of this treatment almost solidified the oil at the concentration used. After six hours' exposure the oil was much more viscous than before treatment, and high doses of this modified oil, which was originally extremely active, failed to promote growth in rats deficient in the fat-soluble factor.

It is evident then that ozone inactivates the fat-soluble factor in active oils and fats. This is in agreement with the recent observations of Hopkins [1920, 1, 2] and Drummond and Coward [1920] that the fat-soluble factor in fats on being exposed to atmospheric oxygen becomes inactivated. The action of ozone is of course much more drastic and therefore more rapid.

I wish to express my indebtedness to Dr J. S. Edkins for having kindly permitted me to use his ozone generator.

A part of the expenses of this research was defrayed from a grant made by the Medical Research Council to whom my thanks are due.

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LXXII. THE NUTRITIVE VALUE OF LARD.

By JACK CECIL DRUMMOND, JOHN GOLDING, SYLVESTER SOLOMON ZILVA and KATHARINE HOPE COWARD.

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(*Received October 18th, 1920.*)

With Plate XII.

During the last few years a considerable amount of attention has been devoted to the study of the distribution of the so-called fat-soluble accessory factor, or vitamin A, in naturally occurring oils and fats. The results of these investigations tend to show that the oils and fats derived from the animal kingdom are, as a rule, decidedly richer sources of this essential dietary constituent than those prepared from vegetable sources. Most authorities, however, regard lard as an exception, having found it practically devoid of vitamin A. Quite early in the study of the growth-promoting vitamins McCollum and Davis [1913] observed that butter fat is of much higher nutritive value for growth than lard.

A similar conclusion was reached by Osborne and Mendel [1913], who suggested that the difference might be ascribed to the fact that lard is a fat derived from storage depôts, whereas butter fat is a product of the synthetic processes of the mammary gland. Many later workers have confirmed these experimental results, and lard has usually been regarded as of little or no value as a source of the fat-soluble A factor.

When one considers the enormous quantities of lard which are prepared for edible purposes at the present time, the importance of ascertaining why lard is thus deficient will be realised. So far as we are aware, no systematic attempt to solve this problem has been made; and only one reference bearing on this question has been encountered in the literature.

Osborne and Mendel [1915] refer to one experiment which they carried out in order to ascertain whether the inefficiency of lard is due to the technical processes to which the fat of pigs is subjected in preparation for the market. Pig fat, direct from the slaughter-house, was finely divided and filtered through filter paper at a temperature just above its melting-point. The filtered product, which they termed "Laboratory Lard," was found to be as inadequate for growth as the commercial products. This result led the authors to conclude that the inferior nutritive value of lard is not due to the heating

which the fat may have received in the course of preparation. This experiment must, however, be regarded as of little value, since apparently no test of the growth-promoting power of the unheated pig fat was made.

In view of the very great importance of the question of the food value of lard, we decided to subject the matter to an experimental study, particularly since it had been observed by Drummond and Coward [1920, 1] that some specimens of raw pig fat contain appreciable amounts of the vitamin A.

Since it is now experimentally proven that the mammalian organism does not possess the power to synthesise the vitamin A, and that it is dependent on its diet for supplies of this essential factor, we concluded that the investigation must proceed along two lines, first, a study of the influence of the diet of the pigs on storage of the vitamin in the fat depôts, and secondly, an investigation of the influence of the technical processes of lard manufacture on the vitamin when present in the pig fat.

The first series of experiments was carried out at the farm attached to University College, Reading, and we desire to thank Prof. Pennington, the Director of the farm, for granting us facilities for the work. The rat feeding tests were carried out at University College, London.

I. Influence of diet of pigs on the vitamin content of pig fat.

Experimental.

A litter of Berkshire pigs farrowed in the early part of 1920 was selected for this experiment. With the exception of one pig, which was a weakling, all the animals were strong and healthy. They were permitted to remain with the sow until weaned at the age of eight weeks, when they were given a ration of toppings (wheat pollards) and whey.

At the age of nine and a half weeks they were divided into five groups. Groups I–IV, which were to be placed on controlled diets, each contained a hog and a sow, whilst Group V, which were the real controls, contained two hogs.

Groups I and II were kept in large, well-designed, stone-floored experimental styes, since it was intended to give them a diet deficient in vitamin A. Groups III and IV were kept in a form of moveable pen improvised by one of us, which permitted the animals to have access to a new plot of green pasturage every day. Group V were reared in the usual manner employed on this farm, and not only received a mixed diet, but also had free run of a small grass paddock.

Group I were given a seriously deficient diet, namely one of toppings and a "synthetic whey." This latter constituent was prepared from caseinogen, lactose, olive oil and salt mixture so as to represent the composition of the whey used in the other experiments, as determined by frequent analysis.

Experiments on rats indicated that this diet was almost devoid of all three

vitamins A, B and C. It was not, therefore, considered likely that the pigs would thrive at all on the food mixture. To our astonishment both animals showed excellent increments of weight for a considerable period of time.

Fig. 1 shows the growth of the hog of this series. It will be seen that the initial growth is considerable in spite of the deficient nature of the ration. Ultimately the animals in this group showed retardation of growth, and lost their healthy appearance. We do not, however, intend to discuss this side of the experiments at this point since we have made a number of other observations on the growth of pigs on deficient diets which will form the subject of a later communication.

Group II were fed on a food mixture of similar composition to that employed for Group I, but the whey was the natural product. Tests of the whey made on rats indicated that it contained insignificant traces of the factor A.

Group III received a basal diet identical with that given to Group II, namely whey and toppings, but in addition were allowed to have an unlimited supply of fresh green food, an addition which they welcomed and of which they made good use.

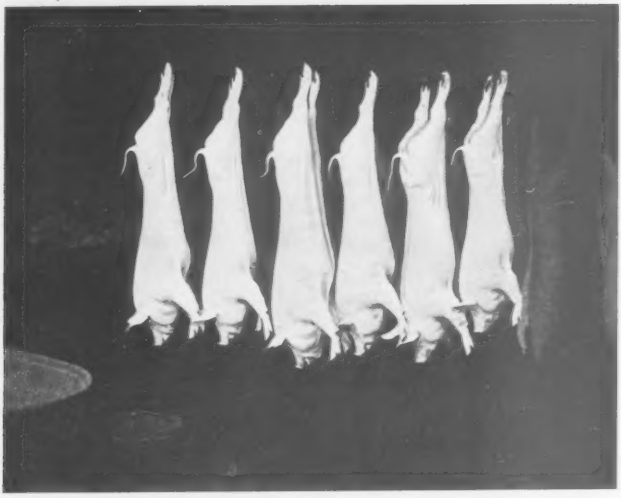
Group IV received no whey, and were confined to a diet of toppings and green foods. Group V received a well-balanced and varied farm diet, including ample grass.

Growth curves of one animal (hog) from each group are given in Figs. 1–5, but, as we have remarked above, we do not intend to discuss this side of the investigation here. Records of food intake were made daily in the case of Groups I–IV, and frequent analyses of the toppings and whey were carried out to control the intake of nutrients. Attached to the growth curves are diagrams indicating the average daily food consumptions at various periods of the experiments.

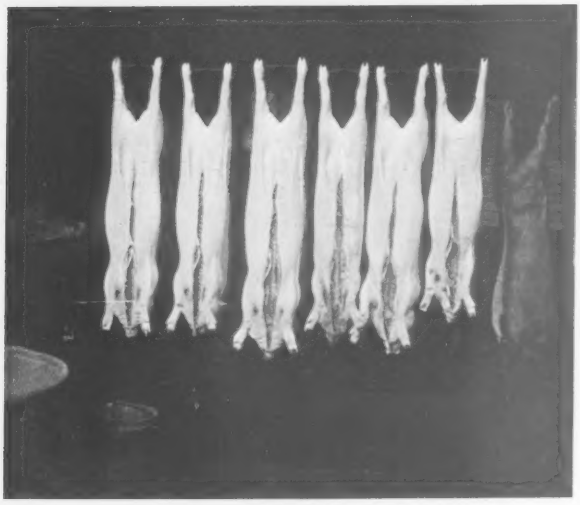
At the end of nearly three months on the experimental diets, the condition of the hogs on the deficient diets 1 and 2 and the size of the control animals led us to conclude that a suitable point had been reached for testing the body fats for storage of the factor A. In view of the fact that our material was limited, we decided to slaughter the hogs of each group only, retaining the sows for the purpose of other observations which we desired to make. Accordingly this plan was carried out.

The hogs were sent to the butcher 24 hours before being slaughtered. A post-mortem examination did not reveal any noticeable abnormality in the organs of the animals. After the carcases had been "dressed" in the usual manner, samples of back fat and perinephritic fat (fleck or leaf) were removed. Photographs of the dressed carcases are given which show the relative sizes of the animals from each group (Plate XII). The small size of the hog fed on the deficient diet given to Group I is obvious, as are the well-developed bodies of the two animals in the control Groups III and V, especially in the latter.

The fats were tested for the presence of the factor A by observing the



1. Side view.



2. Front view.

Photographs of the slaughtered pigs.
From left to right

Group	Sex	Diet	Weight lbs
II	♂	Toppings, whey	122
IV	,,	Toppings, grass	103
V	,,	Full diet with grass	150
V	,,	,, ,,	—
III	,,	Toppings, whey and grass	120
I	,,	Toppings, synthetic whey	84

influence of daily supplements of known weight on the growth of rats, whose growth had been brought to a standstill by a deficiency of that substance.

Small cubes of the fat weighing approximately 1·5 g. were administered to each rat before the daily ration of the basal diet devoid of the factor A

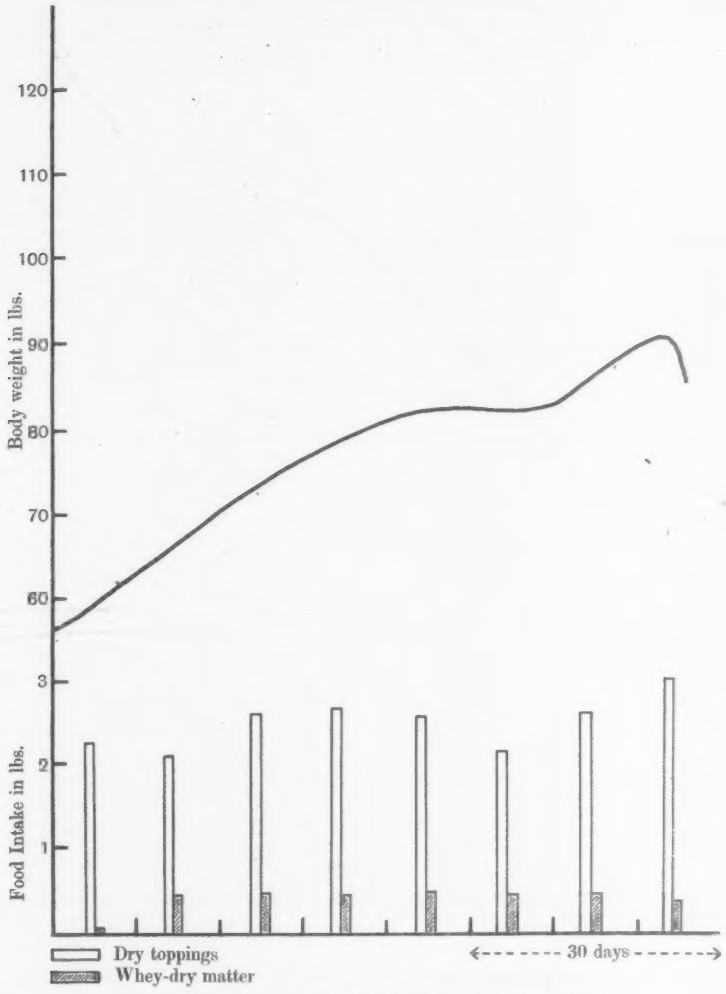


Fig. 1. Growth curve and food intake of hog from Group I.

was given. Practically without exception the whole of the supplements were consumed. The results of these feeding tests are in our opinion quite striking. The body fats derived from pigs in Groups I and II fed on diets deficient in

the vitamin A were found to be of no value as sources of that essential substance for the rat (Curves 1–11, Fig. 6). On the other hand, there is marked evidence that the body fats of the grass-fed animals, particularly those

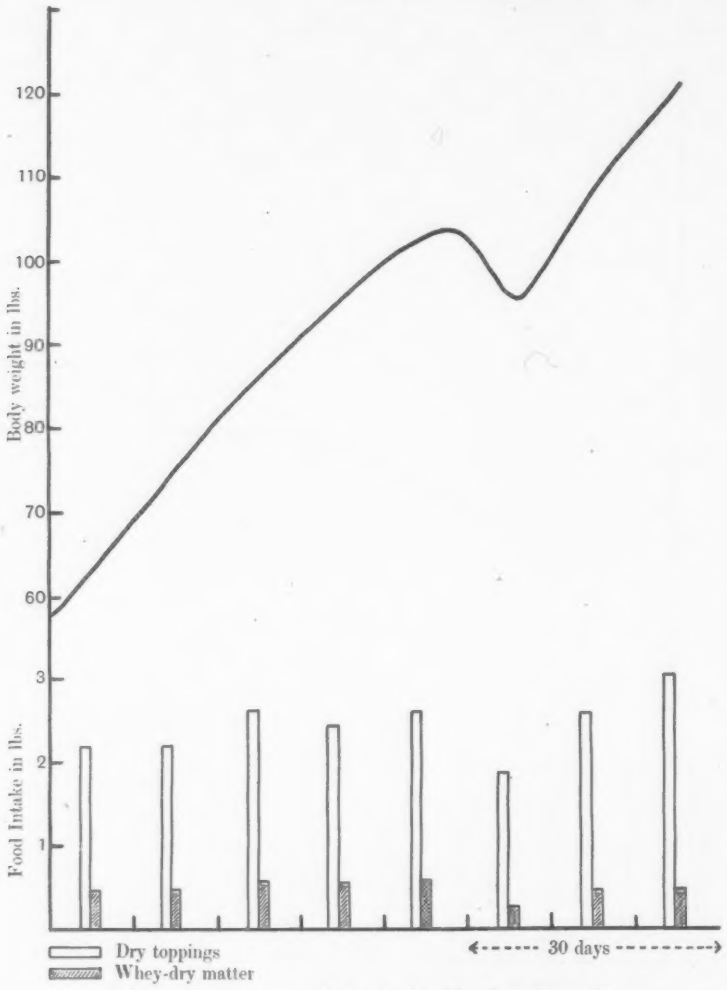


Fig. 2. Growth curve and food intake of hog from Group II.

receiving a mixed farm ration in Group V contained appreciable amounts of the important growth-promoting accessory substance (Curves 12–28, Fig. 6).

The fats from the abdominal cavity and from the subcutaneous deposits were tested separately in each case.

These experiments demonstrate that storage of the vitamin A will occur in the body fat of the pig, provided that the animal receives a diet containing considerable amounts of that substance. This finding is of importance since

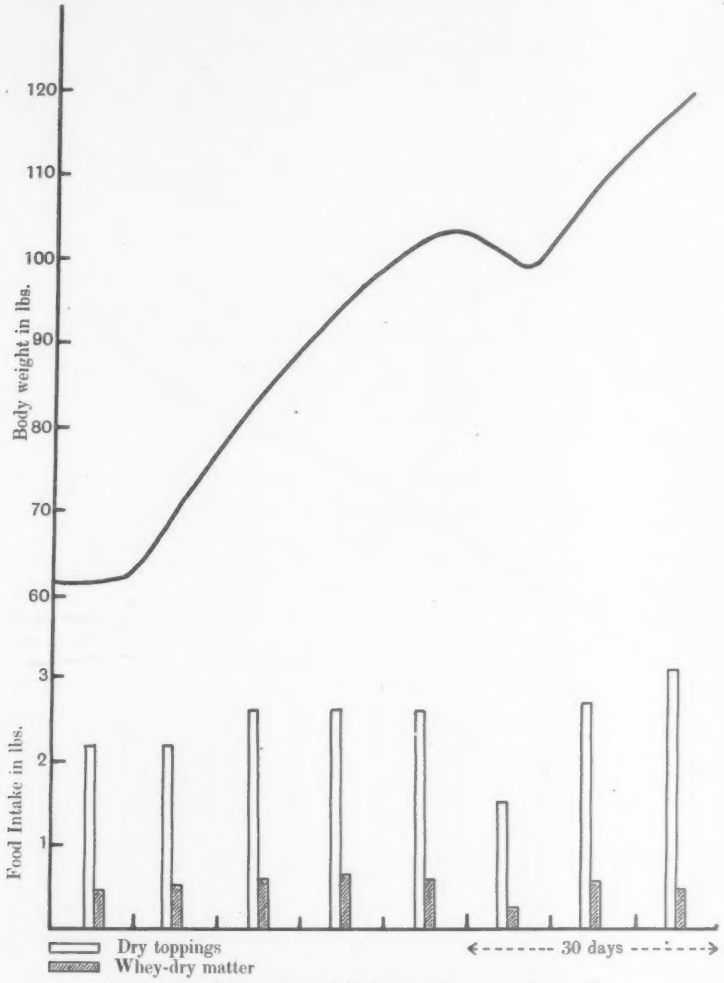


Fig. 3. Growth curve and food intake of hog from Group III.

it shows that the pig is not an exception to the rule that storage of the fat-soluble vitamin A in the fat depôts occurs in animals under suitable conditions.

It does not appear that pig fat is, weight for weight, as rich in vitamin A as is the body fat of other animals fed on a similar type of diet. This may,

however, be due to the fact that the mass of adipose tissue is so much greater in the former species, and that the concentration of vitamin per unit weight of fat would tend to be smaller than in a species such as the cow, where fat deposition is less marked.

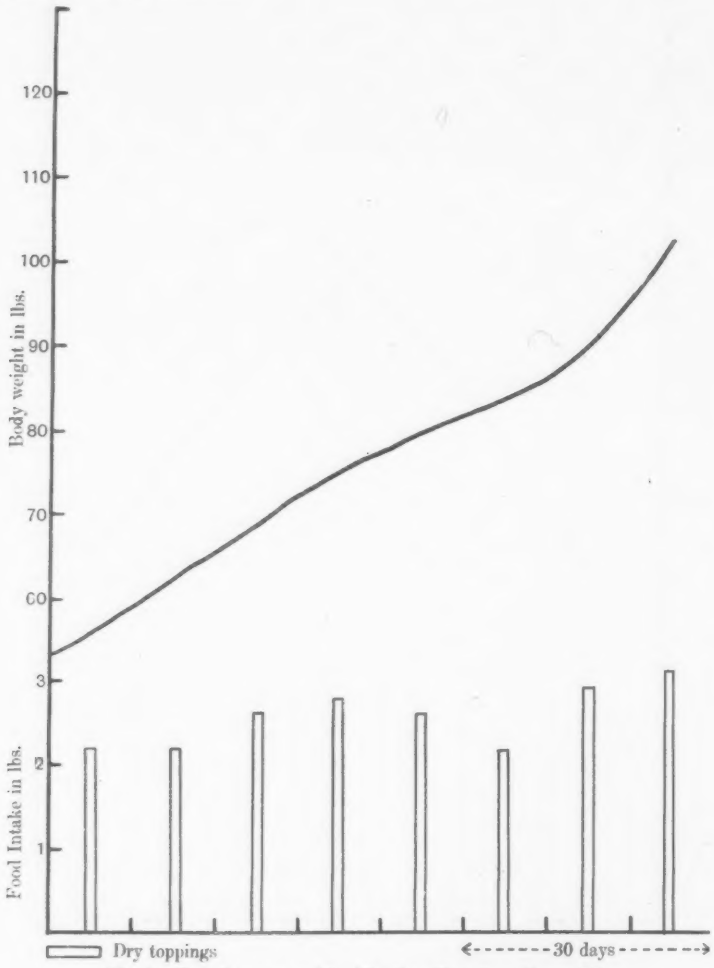


Fig. 4. Growth curve and food intake of hog from Group IV.

II. Influence of the processes employed in the manufacture of lard on the vitamin present in pig fat.

The enormous quantities of pig fat that are converted into lard for human consumption every year may be judged from the fact that in 1912 the total

weight of lard exported from the United States was over 500 million lbs. A considerable proportion of this production is utilised for margarine manufacture, an industry which has grown to many times the size it was in 1912. It is therefore of very great importance to ascertain the influence of the method of preparation employed in lard manufacture on the nutritive value of the fat. Having found by the feeding experiments described above that

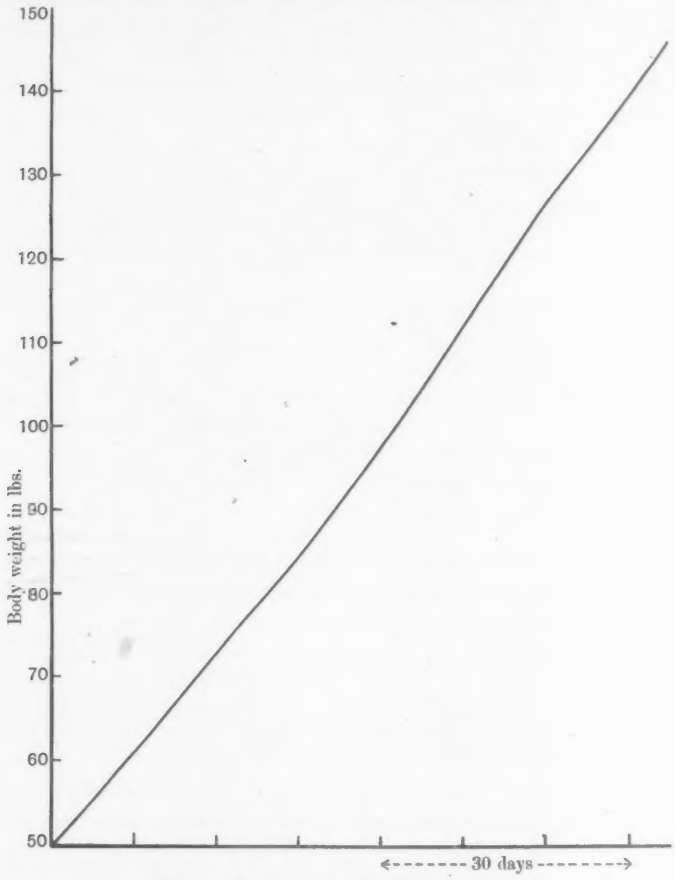


Fig. 5. Growth curve of hog from Group V.

pig fat may contain appreciable quantities of vitamin A when the pigs have been fed on a diet rich in that factor, we proceeded to investigate how the accessory factor is affected by the processes of lard manufacture and refinement. Lard manufacture in this country is not carried out on anything approaching the enormous scale that may be seen in America, and there are

distinct differences between the methods employed there and in this country. The oldest and simplest method of lard preparation still survives in the country parts of England, and we had the opportunity of seeing one preparation by this method. It is only employed by local pig butchers or farmers who handle small numbers of swine, and the amount prepared in this manner is negligible when one considers the total production of lard; the product is largely used for consumption in the immediate locality. This process consists

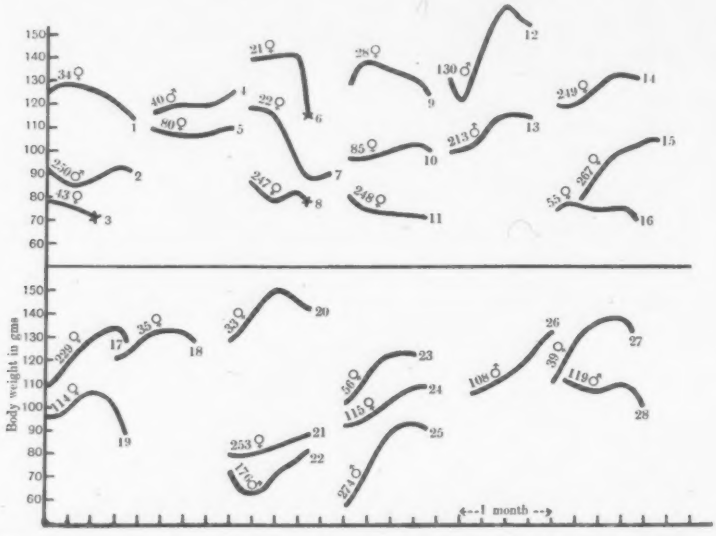


Fig. 6. Growth curves of rats whose diets were supplemented by a daily ration of 1·5 g. fat from the slaughtered pigs.

Rats 1–3 Group I (back fat);
„ 4–5 Group I (abdominal fat);
„ 6–8 Group II (back fat);
„ 9–11 Group II (abdominal fat);
„ 12–13 Group III (back fat);
Rats 14–16 Group III (abdominal fat);
„ 17–19 Group IV (back fat);
„ 20–22 Group IV (abdominal fat)
„ 23–25 Group V (back fat);
„ 26–28 Group V (abdominal fat).

The preliminary period of inhibited growth on the basal ration is omitted.

in heating the pig fat in an open pan over a fire, stirring meanwhile. The melted fat is from time to time skimmed from the surface, reheated to drive off moisture, strained, and poured into bladders. Lard prepared in this manner usually has a brownish tint due to admixture with products derived from the charred tissue. In view of the very small amount of lard which is prepared by this simple farmhouse method, we decided that it was unnecessary to investigate its food value.

For permission to view a large modern lard manufactory in this country we are indebted to Mr R. J. Harris, of the firm of Harris and Co., Bacon Curers, Calne, Wiltshire. To him, and to Mr O. Jones, the chief chemist at

that factory, we wish to express our appreciation of the very kind manner in which they assisted us in our investigation, by placing much information and numerous samples at our disposal. The method employed in this factory is essentially as follows:

The pigs which are drawn from a large area of the surrounding country are slaughtered and the carcases dressed in the usual manner. When cold, the abdominal fat or fleck, which has been stripped and hung up beside the carcase, is minced in a large mechanical mill. The fatty pulp passes into a steam-jacketed pan provided with stirrers, where it is heated to a temperature of about 82°. The fat which separates from the tissues runs off into a second steam-heated pan, where heating at a temperature just above boiling-point (102°) is maintained with stirring for about 10 or 15 minutes to drive off any moisture. The hot dehydrated fat is then clarified either by passing it through a filter press or by allowing it to stand in a settling tank. The lard is now ready to be converted into a suitable solid form and packed for the market. The details of these latter processes have no bearing on the subject of this paper and are therefore omitted.

Very little pig fat other than the perinephritic deposits is used for lard making in this factory, and consequently the product is one of very high standard. The dimensions of the lard industry in the United States have necessitated the adoption of a system of classifying lards. Many of the preparations are made from inferior sources of fat.

The Rules of the Chicago Board of Trade define the following brands of edible lard. (*a*) Neutral Lard No. 1, which is a high quality lard prepared only from the abdominal leaf. (*b*) Neutral Lard No. 2, a similar product prepared from the back fat. These two types are carefully prepared and since they have not been "cooked," they do not keep well. Their chief use is in the manufacture of high class margarines. Only a small amount of lard is prepared by this method in this country. (*c*) Leaf Lard. This is essentially a product which has been prepared by a process similar to the one we have described in detail above. (*d*) Choice Lard or Kettle-rendered Lard, and (*e*) Prime Steamed Lard are products of much lower standard. The latter is indeed usually prepared from the trimmings of the carcases and not from the true fat deposits. They are frequently rendered by the use of high pressure steam, and since they are unpalatable and discoloured in the crude state, they are usually treated by "blowing," or some such process involving oxidation, in order to remove colouring substances and a somewhat unpleasant odour and taste.

We began our investigation by examining an average sample of the pig fat such as is used at Calne for lard manufacture. Curves 4–6, Fig. 7 show that this sample contained an appreciable amount of the fat-soluble vitamin. This confirms the observations by Drummond and Coward [1920, 1] that pig fat may contain the factor A. We also tested a sample of lard which had been made from the same batch of pig fat. This proved to be inactive (Curves 1–3,

Fig. 7) as did several other samples of lard made there on other occasions. Apparently the processes of manufacture were responsible in some manner for the removal or destruction of the vitamin. Naturally the inactivation of the vitamin present in the pig fat will depend on the amount present, which will presumably depend to some extent on the age of the pigs when slaughtered, and to a greater extent on the diet upon which they had been reared.

It was impossible to obtain precise information regarding the diet which had been given to the pigs from which the samples of fat ultimately made into lard were taken. We were informed, however, that it is general in the areas from which the majority of the pigs are drawn to supplement the usual type of diet of toppings, barley meal, whey, etc. with kitchen refuse and green foods. Grass feeding of swine is not carried out to any large extent in this country.

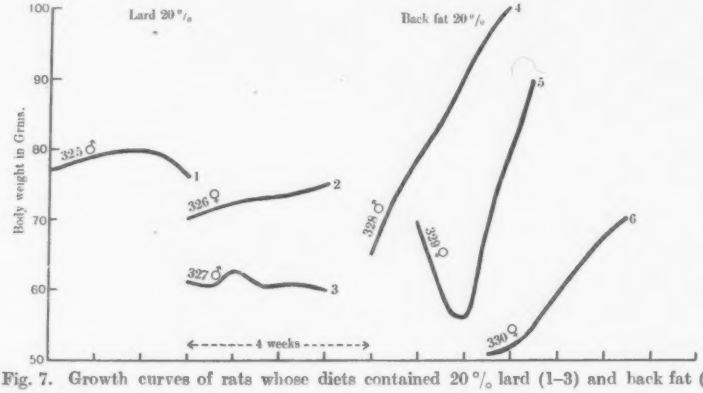


Fig. 7. Growth curves of rats whose diets contained 20 % lard (1–3) and back fat (4–6) respectively.
The preliminary period of inhibited growth on the basal ration is omitted.

The cause of the loss of vitamin during lard manufacture is in our opinion largely due to oxidation, since it has recently been shown that the factor is rapidly inactivated at high temperatures by air or oxygen or by ozone in the cold [Hopkins, 1920, 1, 2; Drummond and Coward, 1920, 2; Zilva, 1920].

The concentration of vitamin A in pig fat, even in grass-fed animals, appears to be considerably lower than in fat derived from grass-fed cattle, and much lower than that usually found in butter, so that when it is remembered that butter fat exposed over a large surface to air at temperatures about 100° may be inactivated in a time as short as one or two hours, it can be understood how the pig fat loses its accompanying vitamin during the heating and stirring used in its conversion into lard.

Recently a paper has appeared in which Daniels and Loughlin [1920] claim to have examined samples of lard which showed considerable growth-promoting power. Not only good growth, but reproduction and satisfactory

rearing of the young were accomplished by their rats when their diet contained 28 % of a commercial preparation of lard. It is possible that the sample with which they worked was one prepared from a pig fat of very rich vitamin content, and that appreciable amounts of that factor had escaped destruction.

Since this communication was completed a sample of lard prepared at Calne from some very active back fat has been examined by us and has been found to possess some activity although not nearly as pronounced as that observed by Daniels and Loughlin. This suggests that some of the fat-soluble vitamin may remain in the lard after treatment in certain cases.

Summary.

1. The pig is able to store up supplies of vitamin A in the body fat when fed upon a diet containing ample supplies of that factor, as for example when grass-fed.

2. When the diet of the pig is deficient in vitamin A, as for example when the diet consists almost entirely of toppings and whey, no appreciable amounts of that dietary factor can be detected in the body fat.

3. The processes employed in the manufacture of lard on a large scale in this country cause a very marked destruction of the vitamin present in the pig fat.

4. The low nutritive value of lard is therefore believed to be due to two causes. First, the diet usually given to fattening pigs in this country is seldom rich in vitamin A, so that the average sample of pig fat contains little or none of that substance; secondly, the processes of lard manufacture undoubtedly cause the destruction of much of the vitamin present in the original pig fat, probably owing to the exposure of the fat to oxygen at high temperature.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

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LXXIII. THE IODIMETRIC ESTIMATION OF SUGARS.

By JULIAN LEVETT BAKER and
HENRY FRANCIS EVERARD HULTON.

(*Received October 18th, 1920.*)

In the course of an enquiry on the iodimetric estimation of sugars, Hilda M. Judd [1920] examined the methods of Romijn [1897], Colin and Lièvin [1918], and Willstätter and Schüdel [1918]. The principle involved in these processes is the oxidation of the sugar to the corresponding monobasic acid by means of iodine in alkaline solution. In the first two methods it was found by Miss Judd that the sugar was not oxidised quantitatively; the third method, however, was adopted by her as it afforded good results. We also found it accurate and rapid.

Briefly the method consists in adding 20 cc. of *N*/10 iodine and 30 cc. of *N*/10 soda to 0·1 g. of the sugar dissolved in 10 cc. of water. The mixture is left at the air temperature for 15 to 20 minutes, then acidified with *N*/1 H_2SO_4 and the excess of iodine titrated with *N*/20 thiosulphate using starch solution as indicator. The reaction in its simplest form may be represented in the case of a hexose as proceeding according to the equation

$$C_6H_{12}O_6 + I_2 + 3NaOH = C_5H_{11}O_5COONa + 2NaI + 2H_2O$$

from which it will be found that 1 g. of the anhydrous hexose reacts with 1·411 g. of iodine, or 1 g. of anhydrous biose, such as lactose or maltose, with 0·743 g. of iodine. How closely this theoretical iodine gram-equivalent is reached in practice will be seen from the figures given in Table I.

In practice an excess of iodine and alkali is employed beyond that required by the equation; for 0·1 g. of sugar, 20 cc. of *N*/10 iodine and 30 cc. of *N*/10 NaOH are taken, although theoretically the necessary iodine for the reaction is present in 11 cc. of the decinormal solution when the sugar is a hexose or in 6 cc. for a biose sugar. Attention may be drawn to the following points.

(1) Our experiments show that it is essential that the reacting solutions should be mixed in the order of sugar, iodine, alkali. If the iodine and alkali are mixed prior to the addition of the sugar a lower iodine value is obtained, due probably to the loss of oxygen from the sodium hypoiodite.

(2) The time required for the oxidation mentioned by Willstätter and Schüdel and adopted by Miss Judd is 15 to 20 minutes; we find, however, that 3 to 5 minutes suffice for the completion of the reaction.

Working under the conditions described we obtained the following results:

Table I.

Sugar	Gram-equivalent		H. M. Judd's results
	Found	Calculated	
Dextrose ($C_6H_{12}O_6$) ...	1·41	1·411	1·315
Levulose ($C_6H_{12}O_6$) ...	0·10	—	0·103
Galactose ($C_6H_{12}O_6$) ...	1·37	1·411	1·418
Sucrose ($C_{12}H_{22}O_{11}$) ...	0·02	—	0·01
Maltose ($C_{12}H_{22}O_{11}$) ...	0·746	0·743	0·7456
Lactose ($C_{12}H_{22}O_{11}$) ...	0·762	0·743	1·502
Raffinose ($C_{18}H_{32}O_{16}5H_2O$)	0·05	—	—
Soluble starch	0·06	—	—

The anhydrous dextrose used had a specific rotatory power of $[\alpha]_D + 53{\cdot}21°$ after crystallisation from alcohol. The galactose had $[\alpha]_D + 80°$ and levulose recrystallised from alcohol had $[\alpha]_D - 88{\cdot}4°$ and a reducing power with Fehling's solution, calculated as levulose, of 99 %. The maltose and sucrose we employed were chemically pure; the former sugar as used for the determination contained 1 mol. of water which has been allowed for in the iodine equivalent recorded in the table. It will be noted that the gram-equivalent of iodine found by us for lactose differs very markedly from the value recorded by Miss Judd. We worked with four separate specimens of carefully purified lactose hydrate and in over a dozen determinations found figures similar to those recorded in the table. We believe, therefore, we are justified in concluding that lactose behaves with iodine in the same way as maltose and gives practically a quantitative yield of the monobasic acid. Consequently, there is no reason to suppose, as suggested by Miss Judd [1920], that lactose is hydrolysed and that both the glucose and galactose molecules are oxidised.

The citation by Miss Judd of Lobry de Bruyn and van Ekenstein's results [1896] in this connection would only be justified if the complete hydrolysis of lactose under the influence of cold alkali were effected in a few minutes, a transformation which was never obtained by these chemists. To settle the point we added alkali to solutions of lactose, maltose and levulose for a period of 20 minutes before the addition of iodine which was then allowed to act for the usual time. Under these conditions we should have obtained even more evidence of the Lobry de Bruyn transformation had it occurred, in the form of a higher gram-equivalent of iodine, but the values obtained were identical with those found when working under normal conditions.

In the course of this work we attempted to ascertain something of the nature of the mechanism of the iodimetric oxidation of carbohydrates and to this end made a series of determinations of the rate at which oxidation took place. Fifty per cent. of the total iodine we found was used up in the first five seconds and the reaction is in all cases complete within five minutes in the cases of dextrose, maltose and lactose. On investigating the values thus obtained by the customary formulae we found that the reaction is not mono-, di- or tri-molecular.

The fact that the time necessary for the reaction as employed by Willstätter and Schüdel [1918] can be reduced from 20 to 5 minutes considerably enhances the usefulness of the method and makes it as convenient as the volumetric Fehling reduction, while an accuracy of 1 % in the case of maltose and lactose and 0·5 % with dextrose and galactose is obtainable.

We applied the method to the analysis of the products of the action of diastase on starch and found that iodine does not differentiate between free maltose and that supposed to exist as "maltodextrin" in a state of combination with dextrin. When precipitated malt diastase was allowed to act on soluble starch for three hours at 50°, the conversion products had the constants $[\alpha]_D + 156{\cdot}5°$ and R, by Fehling's solution, 85·8 % of maltose. A mean of six iodimetric oxidations showed 85·4 % of maltose. From this it is evident that dextrin is unattacked by hypoiodite and that the whole of the maltose present both free and combined is quantitatively oxidised. When starch conversion products are fractionated with alcohol the iodine values agree with the apparent maltose content as found by Fehling's solution. Soluble starch and α-amylodextrin [J. L. Baker, 1902]—the dextrin obtained from starch when hydrolysed with barley diastase—are unacted on by an alkaline solution of iodine.

In the results recorded above solutions of pure sugars were employed. It should, however, be borne in mind that proteins are oxidised by iodine, thus egg albumin and Witte peptone have iodine gram-equivalents of 0·53 and 0·58 respectively. If any intention be entertained of employing the method for the analysis of solutions other than those of the pure sugars, the possible disturbing effects of protein and impurities will have to be considered.

Note added November 15th.

Since writing the above paper our attention has been drawn to a communication by N. Bland and L. L. Lloyd [1914]. These authors have examined (*inter alia*) the behaviour of the sugars towards *neutral* sodium hypoiodite and find that the oxidation of maltose, dextrose and lactose proceeds quantitatively to the corresponding monobasic acids in five minutes at ordinary temperature; sucrose and levulose were unaffected. The neutral hypoiodite used was obtained by mixing equal volumes of $N/10$ iodine and $N/10$ caustic soda.

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LXXIV. THE "AMMONIA COEFFICIENT" OF PREGNANCY.

By WINIFRED CLARA CULLIS and EVELYN E. HEWER.

From the Physiological Laboratory, London (Royal Free Hospital) School of Medicine for Women.

(*Received September 21st, 1920.*)

A study of the nitrogen distribution in the urine during pregnancy has led to the view that the "ammonia coefficient[1]," although it shows a tendency to rise above the normal, should not differ from it very markedly. Indeed it has been claimed by some obstetricians that a value of 15 means a serious departure from a normal pregnancy, and that a value of over 30 is a sign that the pregnancy should be terminated.

The following case is given as a contribution to the study of nitrogen metabolism in pregnancy because it showed a high ammonia coefficient and yet had a successful termination. It is to be regretted that other observations on respiratory values and blood reactions were not made at the same time, as they would have added considerably to the value of the observations. Further, no attempt was in this case made to differentiate between the ammonia and the amino-acids, the "ammonia" being determined by the Folin method. Nevertheless, the figures obtained from the urinary analysis are sufficiently striking to be worth recording[2].

The subject, Mrs W., was a primipara aged 22, who was admitted to the Royal Free Hospital for prolonged vomiting. She was found to be pregnant, retroversion was corrected, and then as the vomiting continued in spite of dieting she was transferred to the Marlborough Maternity Hospital, Endsleigh Street, under charge of Lady Barrett, with a view to induction. Lady Barrett's resident medical officer was Dr Dorothy Chick, to whose keenness and interest we are indebted for the collection of the specimens over the long period before parturition, during which we were enabled to make the analyses at the school laboratories. This was only one of the cases in which Dr Chick's interest in the scientific aspect of her work was manifested, but it was the last before her untimely death in the influenza epidemic of 1919. In publishing these

[1] By the "ammonia coefficient" is meant the ratio between the ammonia and the nitrogen excreted in the urine, taking the nitrogen as 100. The usual way of determining is to estimate the percentage of ammonia and of nitrogen in a 24-hours' specimen of urine: this will give an average coefficient of from 4 to 8, most often about 5 or 6.

[2] All the analyses in this investigation were done by one of us (E.E.H.).

results it is only fitting to record once again our keen sense of the loss that all her colleagues sustained in the death of this particularly able young medical woman, with her scientific attitude towards the problems of work, an attitude strengthened and made valuable by her previous training at Cambridge.

The first specimen of urine in which the ammonia coefficient was determined gave the extraordinarily high value of 70. For other reasons immediate induction was not considered essential, and therefore in view of the urinary analysis we suggested that possibly the high value was in part due to an acidosis of starvation, and particularly to protein deficiency. That the high coefficient could not be explained only by fasting is shown by the fact that in fasting the ratio may rise up to 35, but probably not higher. However, as a trial, the patient was put on to a diet containing much more protein, and almost at once she began to improve.

The volume of urine passed increased (see accompanying charts), and the ammonia coefficient dropped rapidly (although still high). Intermittent vomiting continued for some time, but the patient looked and felt much better. Two months after admission to the Maternity Hospital she was discharged, with instructions to report at out-patients, and to bring fortnightly twenty-four-hourly samples of urine.

Two months later she was readmitted for vomiting, and a trace of albumin in the urine, but was discharged a week later much improved.

Six weeks later she was readmitted for confinement. The baby was born slightly jaundiced, and weighed $5\frac{3}{4}$ lb. ♀. The patient was discharged doing well, and feeding the baby.

In view of the interest of the case, she was instructed to report periodically, and to bring samples of urine with her. From these we have been able to draw up a chart showing her urinary excretion when not pregnant; the analyses give a normal ammonia coefficient, a very high urea average, and a small volume of urine.

At the present time she is again pregnant, and the urine is being systematically analysed: up till the present nothing abnormal has been detected.

A study of the accompanying tables and charts will show the way in which the ammonia coefficient varied with the condition of the patient. It is particularly unfortunate that no urinary analyses were made just before and after confinement: this was due to the serious outbreak of influenza at the Hospital, during which Dr Chick herself died.

The following points appear from the charts:

1. The patient has no inherent abnormality of metabolism, the coefficient being of the average physiological value, *i.e.* from 4 to 8.

2. The high ammonia percentage was not due to concentration of the urine.

3. There was a very definite retention of nitrogen.

4. There was a definite parallelism between the daily total NH_3 and daily

Table I. *Urinary Analysis (Mrs W., Non-pregnant condition)*

Date	Quantity oz.	S.G.	Titration acidity %	NH_3 %	N %	Urea %	NH_3 Coeff.	Total NH_3	Total N	Total Urea
Mar. 16–17	—	1023	71·1	0·1	1·6	2·75	6·2	—	—	—
Sept. 7–8	—	1030	58	0·15	1·81	3·2	8·3	—	—	—
Sept. 21–22	30	1030	55·1	0·12	1·78	3·85	6·7	1·02	15·1	32·8
Sept. 29–30	—	1030	53·8	0·12	1·73	3·8	6·9	—	—	—
Nov. 21–22	36	1021	24·8	0·074	1·26	2·2	5·9	0·76	12·8	22·5
Dec. 2–3	22	1027	12	0·064	1·13	1·92	5·6	0·4	7·1	11·98

In this table and in Table II

Titration acidity — Expressed as number of cc. of $N/10$ NaOH required for 100 cc. urine using phenolphthalein as indicator.

Urea ... ... — Expressed as grams. Estimated by hypobromite method.

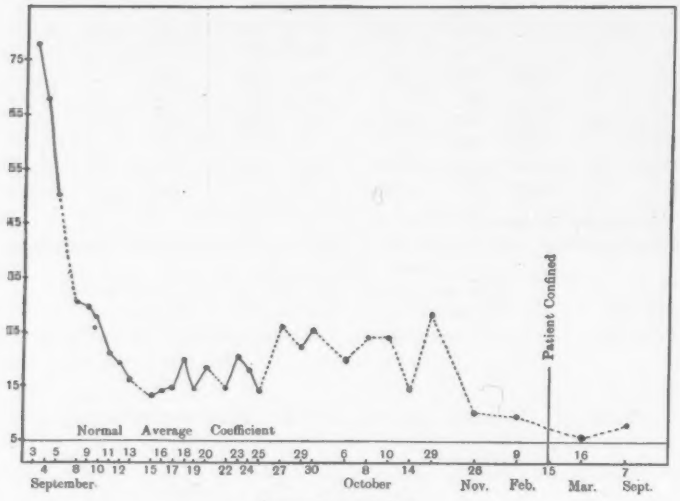
Nitrogen ... — Expressed as grams. Estimated by Kjeldahl method.

Ammonia ... — Expressed as grams. Estimated by Folin's formaldehyde method.

Table II. *Urinary Analysis (Mrs W., Pregnant condition)*

Date	Quantity oz.	S.G.	Titration acidity %	NH_3 %	N %	Urea %	NH_3 Coeff.	Total NH_3	Total N	Total Urea
Sept. 3–4	8¾	1022	33·3	0·67	0·86	—	77·9	1·66	2·1	—
4–5	9	1017	48	0·7	1·03	—	67·9	1·79	2·6	—
5–6	9	1019	78·5	0·54	1·08	—	50	1·38	2·8	—
8–9	16	1016	30	0·23	0·76	—	30·25	1·04	3·45	—
9–10	20	1015	24·7	0·22	0·74	—	29·7	1·25	4·2	—
10–11	20	1016	21·4	0·17	0·61	—	27·8	0·96	3·46	—
11–12	44	1012	17·6	0·12	0·57	0·9	21	1·5	7·1	11·23
12–13	23	1012	26·6	0·12	0·62	1·1	19·35	0·78	4·04	7·2
13–14	30	1016	28·1	0·13	0·77	1·2	16·8	1·11	6·55	10·2
15–16	30	1005	12·85	0·05	0·38	0·5	13·2	0·43	3·2	4·3
16–17	45	1009	30·9	0·07	0·5	0·8	14	0·89	6·4	10·3
17–18	25	1012	34·3	0·09	0·62	0·8	14·5	0·64	4·4	5·7
18–19	25	1010	50	0·1	0·5	0·8	20	0·71	3·5	5·7
19–20	30	1007	22·85	0·08	0·54	0·8	14·8	0·68	4·6	6·8
20–21	36	1011	37·6	0·1	0·54	0·75	18·5	1·02	5·5	7·7
22–23	40	1008	17·1	0·08	0·54	0·7	14·8	0·91	6·12	7·9
23–24	39	1010	41·9	0·11	0·54	0·65	20·3	1·22	5·97	7·2
24–25	28	1011	34·2	0·11	0·61	0·8	18	0·87	4·84	6·3
25–26	29	1008	9·1	0·06	0·43	0·52	13·95	0·49	3·54	4·3
27–28	7	1018	49·5	0·21	0·82	1·45	25·6	0·42	1·63	2·9
29–30	+	1009	13·8	0·09	0·4	0·55	22·5	—	—	—
Oct. –1	27	1015	61·4	0·2	0·8	1·3	25	1·53	6·13	10
6–7	15	1016	37·1	0·14	0·73	1·2	19·18	0·6	3·1	5·1
8–9	27	1012	52·8	0·15	0·63	1	23·8	1·15	4·8	7·7
10–11	25	1014	59	0·19	0·8	1·37	23·75	1·35	5·7	9·7
14–15	30	1013	2	0·07	0·5	0·62	14	0·6	4·25	5·3
29–30	—	—	9·5	0·23	0·8	0·8	28·7	—	—	—
Nov. 26–27	—	—	17·5	0·07	0·66	0·85	10·6	—	—	—
Feb. 9–10	—	1016	18·5	0·07	0·71	1·25	9·8	—	—	—
15	Patient confined									
Mar. 16–17	—	1023	71·1	0·1	1·6	2·75	6·2	—	—	—

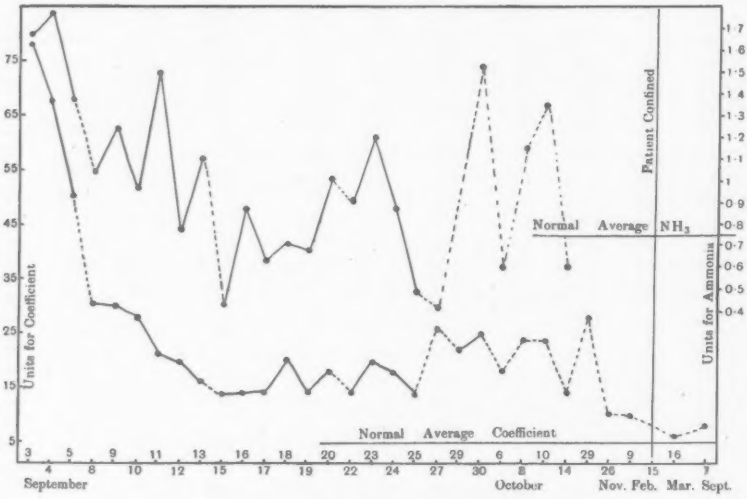
Chart 1



Ammonia coefficient

$$\text{i.e. } \frac{100 \times \text{Percentage of } NH_3}{\text{Percentage of N}}$$

Chart 2

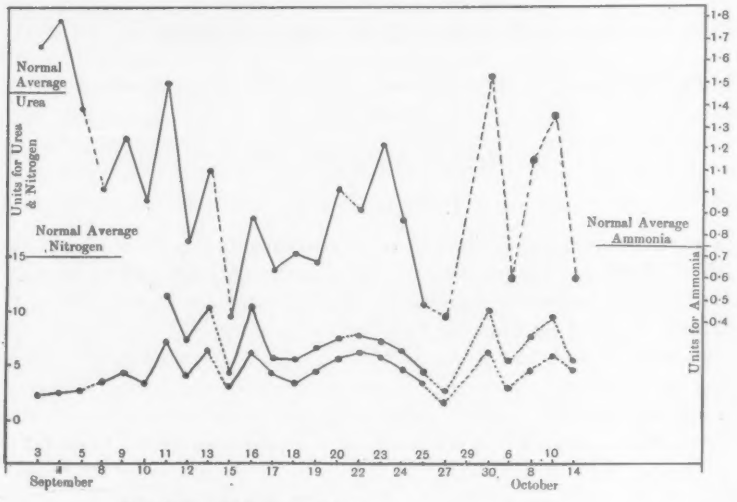


Top Curve—Total NH_3 excreted in 24 hours
Lower Curve—Ammonia coefficient

total urea: comparison with a normal chart shows that increased NH_3 in the usual way means decrease of urea (on a constant diet): in this case, increase of NH_3 went with increase of urea.

5. There appeared to be no relationship between the vomiting and the coefficient: the latter was much more dependent on the diet. (It is, of course, well known that starvation raises the coefficient.)

Chart 3



Top Curve—Total Ammonia
Middle Curve—Total Urea
Lower Curve—Total Nitrogen
} Excreted in 24 hours

6. There was no relationship between the total NH_3 excreted and the NH_3 coefficient.

Quite recently another case of abnormally high ammonia coefficient has been met with, in connection with an investigation that is being carried out at the London (R.F.H.) School of Medicine for Women, on the metabolism of pregnancy under the Medical Research Council's Scheme of Child Life Investigation.

We should like to thank Lady Barrett for permission to publish this case.

LXXV. THE PENETRATION OF ELECTROLYTES INTO GELS. II: THE APPLICATION OF FOURIER'S LINEAR DIFFUSION LAW.

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I. Introduction.

The ways of studying diffusion can be divided roughly into three classes, two of which, electrical measurements and quantitative analysis, may be described as the standard methods of determining diffusion rates; the third experimental procedure, marked off from the first two by its simplicity, has been named the indicator method. The characteristic of simplicity is appreciated on looking at the list of essential apparatus—a rack of test-tubes and a ruler. The tubes are filled half full with a gelatin solution containing some indicator, and after the formation of a solid jelly a solution of the substance under investigation is poured in and a colour change in the indicator demonstrates the forward march of the diffusing substance. When this is a neutral salt the change consists of the formation of a precipitate with the reagent placed in the jelly.

This method has been investigated recently by von Fürth and Bubanović [1918]. Their research on the quantitative aspects of the problem is a notable advance, but their method of calculation of the diffusion coefficient (defined below) merely gives ratios and not absolute values. This is a serious drawback, and Professor Stiles, who is revising the indicator method [1920], urged me to attempt the calculation if there was the slightest hope of success. The winning of new results is beyond the scope of this essay. It is limited to the effort to work out a theoretical formula free from arbitrary constants, and secondly the illustration of the method of using this formula by which new results can be won.

The mathematical expression arrived at is general, therefore it does not matter what substance is used in the experimental testing—accordingly it will be put to the trial with the familiar salt, sodium chloride. Anyone reading this account will see how essential is such a test, though unfortunately it expands the writing to an inordinate length. The theory, from the point of view of the laboratory worker, lacks the charming simplicity of the experimental technique.

This disadvantage is inevitable—in fact, it must be confessed that in the beginning, the prospect of working out the result looked small indeed. The given measurement was doubtless a function of the diffusion coefficient "κ," but it was a function also of about a dozen other variables. The complexity of the theory might easily have discounted the advantages of the practice, but a review of these advantages had better follow the account of the method of calculation—a method mainly due to a lucky coincidence.

II. Summary of the theoretical principles of diffusion.

Leaving more general aspects, we may now proceed to the special problems of the calculation. The laboratory measurements are given in terms of centimetres of salt penetration. These distances depend on a variety of factors. Firstly, we have to consider the laws of diffusion, which are expressed in the form of differential equations, integrable for certain "boundary conditions," which are the second group of factors to be examined. Essentially they depend on the form of the mass of gelatin and the stirring of the salt solution. Thirdly, there are the phenomena peculiar to the experiment, for example, the temperature, the nature of the diffusing substance and the resistance of the medium.

The theory of the first two of these groups of phenomena belongs to the realm of mathematical physics, and was worked out by Fourier 100 years ago. The last group falls within the province of the experimenter.

The factors of interest to him all affect the diffusion coefficient, κ, so he has to reverse the original procedure, to find what value of κ must be assigned in the formulae to give the observed relation between concentration, time and place. This is easy in the two standard methods, but if an indicator is present, the problem becomes much more complicated, and there is nothing for it but to go back to first principles. The following portion of this section is devoted to the explanation of the terms in the formulae required, and the definition of the conditions under which they hold good. For the explanation of the formulae themselves, it is enough to give a general reference to Fourier's *Théorie Analytique de la Chaleur*, or the modern text-books of the mathematical theory of heat conduction, for example, Carslaw's *Fourier's Series and Integrals*. Even though we need only the simpler formulae it would take far too long and serve no useful purpose to go through the expansion of unity in a sine series—which is needed for formula (I) or Laplace's solution for the flow of heat in an infinite solid, required for formulae (II) and (III).

For our present work they are merely stated in the form most suitable for calculation with ordinary tables.

There is no possibility of avoiding these formulae. Von Fürth and Bubanović's penetration factor can be used for certain purposes, but the overwhelming reasons for calculating "κ" are stated below.

(1) There is no need to introduce an empirical law expressing the effect of concentration, so that the true effects of concentration are laid before us.

(2) We can test the absolute accuracy of the work by comparison with standard methods.

(3) Thirdly, κ must be known if we are to make any practical calculations on the diffusion of salt in the system.

(4) It is the fundamental constant which is of use to us in the study of the phenomena, in which we are interested—free from the complications due to the "boundary conditions."

These reasons for making the attempt to calculate κ are quite decisive. The fundamental law, that the rate of diffusion is proportional to the concentration gradient, was published by Fick as a special case of Fourier's linear diffusion law.

$$\frac{\partial u}{\partial t} = \kappa \frac{\partial^2 u}{\partial x^2}$$

t = time in seconds,
x = distance in centimetres,
u = concentration of salt,
κ = diffusion coefficient.

The diffusion coefficient is the amount of salt in equivalents which would flow across an area of one square centimetre, if the concentration at one point was one equivalent per litre greater than that at another point, one centimetre distant in the line of flow.

This partial differential equation has, of course, many different solutions, depending on the initial and boundary conditions. We will consider first the example which resembles the type of salt penetration of most practical interest. In this example the surface is kept at constant salt concentration by immersion in a large bath of solution.

To define the conditions more accurately, let us call the direction of penetration the "x" axis, and at the surface of the gel let $x = 0$. Suppose the test-tube is 14 cm. long, and distances into it are reckoned positive. Let u = the salt concentration.

Let u_0 be the concentration of salt in the bath of solution. Then we have the following conditions:

$$u = 0, \text{ when } 0 < x < 14 \text{ at } t = 0,$$
$$u = u_0 \text{ at } x = 0,$$
$$du/dx = 0 \text{ at } x = 14.$$

This last condition is represented mathematically by adding another 14 cm. to the length, with the condition that

$$u = u_0 \text{ at } x = 28.$$

Both the differential equation and the boundary conditions are satisfied by the following series of sine and exponentials, where l is double the length of the test-tube:

$$u = u_0 \left\{1 - \frac{4}{\pi}\left(e^{-\frac{\kappa\pi^2 t}{l^2}} . \sin\frac{\pi x}{l} + \tfrac{1}{3} e^{-\frac{9\kappa\pi^2 t}{l^2}} . \sin\frac{3\pi x}{l} + \tfrac{1}{5} e^{-\frac{25\kappa\pi^2 t}{l^2}} . \sin\frac{5\pi x}{l} + \ldots\right)\right\} \ldots . (\mathrm{I})$$

This series converges too slowly to be very helpful in practical work, but with its aid we can define the limits within which a simpler formula will hold good. For this purpose the calculations need not be carried very far and in any case tubes do not agree in length to four places of decimals. Taking Öholm's figure $\kappa = 1{\cdot}07$ per day, and assuming diffusion has been taking place for six days, we find that

$u = 0{\cdot}0007N$ if the calculation includes the fourth term,

$u = 0{\cdot}0003N$ if five terms are summed.

The amounts are so small that we shall probably not exceed the error in calculations with four figure tables if we assume that no salt has reached the end of the tube in the first two or three days of the experiment.

This conclusion greatly simplifies the problem, since the initial and boundary conditions may be re-stated with the assurance that the length of the tube does not enter into the problem, and for purposes of calculation may be taken as infinite.

$$u = 0, \text{ when } x > 0 \text{ at } t = 0,$$
$$u = u_0 \text{ at } x = 0.$$

The solution for these conditions is

$$u = u_0 \left\{1 - \frac{2}{\sqrt{\pi}} \int_0^q e^{-q^2}\, dq\right\}, \quad \text{.....(II)}$$

where $$q = \frac{x}{2\sqrt{\kappa t}}.$$

The formula could be stated more briefly if Kramp's table of the integral between q and infinity were more generally available, but the form given above is used because the probability integral tables are much more likely to be at hand. Given the table, it is the work of a few moments to determine "κ" when "u" is known.

The second type of experiment is carried out as follows. The salt solution is put in a tube half filled with gelatin and diffusion is allowed to take place without any circulation of the solution, so that we have a concentration gradient in the liquid itself.

If, as before, we consider only the first few days, and assume κ is constant, we have the following conditions:

$$u = u_0, \quad x < 0,$$
$$u = 0, \quad x > 0.$$

After reversing the sign of x the solution which satisfies these conditions is

$$u = u_0 \left\{\tfrac{1}{2} + \frac{1}{\sqrt{\pi}} \int_0^q e^{-q^2}\, dq\right\}. \quad \text{.....(III)}$$

III. Negative salt convention.

The formulae stated in the preceding section are well-established in the study of diffusion by the classical methods—they can be used to calculate "κ" directly. The indicator method, however, does not give us the direct

relation between u and u_0 at the point x and at the time t. At the demarcation surface, we could get the concentration of chloride ions from the solubility of silver chloride 0·00015 %, but this does not help us much. It is obvious that the more silver nitrate is streaming up, the more slowly will the precipitate advance, because there will be longer to wait for enough sodium chloride to stream down and precipitate all the silver ions in a given space.

Some method of handling the problem must be devised, and the most promising line of attack seemed to have as base the obvious fact that one silver ion removes one chloride ion from the sphere of action, when silver chloride is precipitated. The development of this idea is to assume that we can call the silver nitrate "negative sodium chloride."

That is to say, a gel with 0·01 g. molecules of the silver salt would be called a $-0{\cdot}01N$ sodium chloride. Making the usual shift in the scale, this is described for the next calculation as 0, and at the zone of precipitation, where the sodium chloride really is 0, the concentration therefore becomes $+0{\cdot}01N$ sodium chloride.

Calling the silver nitrate concentration v_0 and the sodium chloride concentration u_0, we have at the edge of the precipitation the relation

$$u = \frac{v_0}{u_0 + v_0}.$$

This is a known fraction of u_0, and therefore we can find $q = x/2\sqrt{\kappa t}$ from the tables of the probability integral. This formula is only a first approximation, as it neglects the rate of movement of the silver nitrate. Before entering on this secondary complication our policy is to find if the approximation gives us a result which is at all promising, because the next step must obviously depend on whether the percentage error is 10 or 10,000.

IV. Experiments with original technique.

The first data which will be used to test the formula were obtained on July 29th, 1919, by the half test-tube method.

Table I.

Diffusion coefficient of sodium chloride in 1 % agar gel. July 29th, 1919.
NaCl solution poured into half test-tubes of gel.
Concentration of silver nitrate $0{\cdot}005N$.
κ_1 calculation of "κ" from 1st reading, 19 hours.
κ_2 calculation of "κ" mean of later readings.
$q = x/2\sqrt{\kappa t}$ taken from tables, using formula (III).
The values of κ are given in c.g.s. units $\times 10^5$.

Salt concentrations		$2N$	N	$0{\cdot}5N$	$0{\cdot}2N$	$0{\cdot}1N$	$0{\cdot}05N$
Corresponding "q"		1·98	1·82	1·65	1·39	1·18	0·94
Temp. 22°	κ_1	1·54	1·55	1·54	1·79	2·0	1·84
22°	κ_2	1·7	1·7	1·7	2·08	2·40	2·46
13·6°	κ_1	1·19	1·18	1·21	1·48	1·65	1·75
13·6°	κ_2	1·36	1·27	1·34	1·67	1·85	1·95
0°	κ_1	0·82	0·86	0·87	0·99	1·16	1·42
0°	κ_2	0·88	0·90	0·89	1·13	1·25	1·31

The error is generally nearer 10 % than 100 % in these figures, but it is quite clear that the indicator method is of no use unless it turns out more consistent results than these. Perhaps the most serious discrepancy is the difference between the κ calculated from the first reading and the mean κ. If this is a real physical fact, it would of course be very interesting, but there are other causes we must examine first.

(1) Is the "negative salt" formula too crude?

(2) Is there an error in the experiment?

No. (1) may be dismissed for the time being, as it could not be held responsible for the difference of κ_1 from κ_2. We are left with the second alternative. There are a great many possible sources of error—but some can be ruled out at once, as for instance the suggestion that the dense precipitate obstructs diffusion—such a phenomenon would produce an error in the opposite direction.

The most probable explanation of the discrepancy is that the salt solution was circulating in the test-tubes. The liquid at the gel surface continually loses salt, and, as the heavier solution is above it, mixing is bound to take place. This can be eliminated by a slight modification of the experimental technique.

The original method with the solution above the gel will be called type A. The modified method with tube inverted will be called type B. Type C unlike A and B requires formula (II), and it is theoretically more reliable because the diffusion gradient is in gel throughout, not one-half in gel, the other in water.

V. Data obtained with modified technique.

Some experiments by methods B and C are given in the tables below. They illustrate the theory and are not without value in showing what degree of consistency may be obtained in a first trial, but the numerical results are not very reliable, because the temperature was not controlled.

Table II.

Diffusion of sodium chloride into a 5 % gelatin gel, 0·0201*N* $AgNO_3$. "Depth" in cm. of ppt. "κ" in c.g.s. units $\times 10^5$. Temperature 12°.

Time		*N* NaCl		0·5*N* NaCl		0·1*N* NaCl	
hrs.	mins.	Depth	κ	Depth	κ	Depth	κ
1	0	0·55	0·99	0·5	1·11	0·3	1·06
22	36	2·5	0·92	2·2	0·97	1·3	1·14
25	44	2·8	1·01	2·4	—	1·5	1·15
28	53	3·0	1·02	2·5	0·98	1·9	1·18
35	50	3·7	1·25	3·2	1·26	2·0	0·99
48	16	3·8	0·99	3·3	1·02	2·6	1·08
73	15	4·6	0·96	—	—	—	—

The figures given in this table are a great improvement on those of Table I, with the exception of the readings taken at the 35th hour. The remaining determinations show that κ does not really change with time, and therefore

method A is discredited. The constant of diffusion, however, does vary with the concentration, being greater in the more diluted and therefore more highly ionised solution.

N NaCl	Mean κ	$0{\cdot}98 \times 10^{-5}$	Mean error 3 %,
0·5*N* NaCl	Mean κ	$1{\cdot}02 \times 10^{-5}$	Mean error 4½ %,
0·1*N* NaCl	Mean κ	$1{\cdot}10 \times 10^{-5}$	Mean error 5½ %.

The mean error on a single determination is large, but on the whole the results prove that the indicator method gives reasonable results at the first trial, a most important advantage where a diffusion coefficient must be measured as an incidental portion of a wider research.

The results in Table II were all obtained by method B, so our next step is to give observations, made with the surface of the gelatin kept at constant salt concentration by exposure to a large bath of stirred salt solution.

Table III.

Penetration of *N* NaCl into a gelatin gel, with 0·0201 gram-mol. of $AgNO_3$. Method "C." Depth in cm. of precipitate.

κ = diffusion coefficient in c.g.s. units $\times 10^5$.

Temperature 12°. Time, hours and minutes.

Time	1.0	2.49	4.9	6.0	7.30	11	22.36	25.44	28.53
Depth	0·6	0·9	1·2	1·5	1·62	1·95	2·85	3	3·3
κ	0·92	0·82	0·89	0·96	0·90	0·93	0·93	0·90	0·97
Time	48.16	53.30	73.15	96.20	120.6	197.30	219.0	263.30	
Depth	4·25	4·45	5·23	6	6·7	8·7	9·1	10	
κ	0·96	0·96	0·96	0·96	0·96	0·98	0·97	0·97	

The results in this table are in accordance with those in Table II, and because the method and formula are different, the "negative salt convention" is placed in a much stronger position than if it depended on one method alone; but the figures also prove that we must qualify the claim, made in the beginning, that all the apparatus needed is a rack of test-tubes and a ruler. The distances measured in the first few hours, when the temperature was constant, are so small that the experimental error with a wooden ruler is a large fraction of the whole, and if the experiment is protracted, the cupboard temperature cannot be relied upon. The night temperature would be below 12°. We must add a thermostat to our list of apparatus.

It is not absolutely essential because the heat reserve in a pan of water prevents much temperature variation, as is shown in Table IV, an experiment carried out to test the effect of varying the silver concentration, and also to get a direct comparison between the old method A and the method C, which was adopted for later experiments.

Table IV.

Penetration of 0·1*N* NaCl into 5 % gelatin, with 0·034 gram-mol. $AgNO_3$.

Time	0	17·3 hours	25·2 hours
Temp.	12·0°	12·3°	12·2°
Method A	$\kappa = 10^{-5} \times$	1·35	1·35
„ C	$\kappa = 10^{-5} \times$	1·09	1·04
„ A	depth in cm.	1·95	2·35
„ C	depth in cm.	2·13	2·5

Method A makes "κ" come out much too large, an error we must put down to circulation inside the test-tube. The data for B and C are set forth below, and for comparison a calculation of κ for water is made from Öholm's data [1905] (using his value of the temperature coefficient, in the neighbourhood of 18°, a rise of 2·5 % per degree centigrade).

Table V.

Method B	N NaCl $\kappa = 0{\cdot}98 \times 10^{-5}$	$N/10$ NaCl $\kappa = 1{\cdot}10 \times 10^{-5}$
Method C	0·96	1·06
Öholm	1·06	1·11

The negative salt convention has been subjected to three tests:

(i) Variation in the silver concentration.

(ii) Variation in the sodium chloride concentration.

(iii) Alteration of the boundary conditions. If it was wildly in error, at least one of these tests should expose it, but if we allow for the nightly fall in temperature, the differences from Öholm's figures are about as large as the experimental error. The difference between the four experimental values of κ is in accordance with expectations.

A 5 % gelatin offers some resistance to diffusion, therefore "B" should give bigger values than "C," and the relatively faster movement of the ions causes the more highly dissociated 0·1N salt to diffuse more quickly than normal sodium chloride.

VI. Derivation of corrected formula.

The data given above are sufficient to establish the "negative salt" convention as a first approximation, and for diffusion experiments it is a fairly close approximation. To carry it further requires a considerable extension of the work, mathematical and practical.

The temperature control in particular must be very reliable but it is the former branch of the subject which must be considered here.

There are two difficulties in particular which were omitted in the preliminary treatment. The first of these is ionisation. As a general rule the undissociated molecule diffuses more slowly than the ions, so that other things being equal, the more dilute the solution, the larger is the value of κ. This variation in κ is quite big enough to be noticeable in the results calculated above. Each concentration of salt has a particular value of κ. In the gel the concentration varies from say N to 0. The κ we calculate will be that of some intermediate concentration, probably about $N/2$, but the indicator method does not give us information on this point, so it has perforce to be neglected.

The second and much the more important factor is the rate of diffusion of the silver nitrate. The original convention was that silver nitrate could be treated as negative sodium chloride. This assumption is arbitrary and it is to be feared it offends common sense.

In the following section, an attempt is made to treat the indicator method theoretically without this simplifying assumption. The change of κ with concentration will not be introduced into the calculations and the average value between the two limits will be used. This approximation is unavoidable.

The experimental facts to be considered have been stated before, but they must now be defined with greater precision. The silver nitrate is streaming down the tube. Let its concentration be v and its diffusion coefficient λ, then

$$\frac{\partial v}{\partial t} = \lambda \frac{\partial^2 v}{\partial x^2}. \quad \text{.....(1)}$$

Similarly for the chloride

$$\frac{\partial u}{\partial t} = \kappa \frac{\partial^2 u}{\partial x^2}. \quad \text{.....(2)}$$

At the position a, the demarcation line between the precipitate and clear gel, it is assumed that the concentrations v and u both equal 0. This is not strictly true. Really the product of their concentration is a constant, and it is possible that u for instance might be quite large. This possible complication need not be considered here, as at the boundary it is certain that the chloride concentration must be small. If this assumption is to be true, the amount of salt brought to the point "a" must be equalled by the amount of silver nitrate. Therefore at a

$$\lambda \frac{\partial v}{\partial x} + \kappa \frac{\partial u}{\partial x} = 0. \quad \text{.....(3)}$$

In addition to these equations the following boundary conditions must be satisfied:

$$v = v_0 \text{ when } x > 0 \text{ at } t = 0,$$
$$u = u_0 \text{ at } x = 0,$$
$$u = v = 0 \text{ at } x = a.$$

Let $$\phi_a = \frac{2}{\sqrt{\pi}} \int_0^a e^{-\beta^2} d\beta.$$

Then $\phi\left(\frac{x}{2\sqrt{\lambda t}}\right)$ and $\phi\left(\frac{x}{2\sqrt{\kappa t}}\right)$ are particular solutions of the differential equations (1) and (2). The combination of these two integrals in a general solution is not immediately obvious.

The only method is to make some trial assumptions, and find out what values must be assigned to the constants if the formula is to satisfy the conditions of the problem. The simplest possible guess we can make is that v and u are linear functions of $\phi \frac{x}{2\sqrt{\lambda t}}$ and $\phi \frac{x}{2\sqrt{\kappa t}}$. F. Neumann [Weber-Riemann] has shown that this method can be used successfully in solving a problem with similar equations, the formation of ice on a pool of still water.

Let $$v = A + B\phi \frac{x}{2\sqrt{\lambda t}},$$
$$u = C + D\phi \frac{x}{2\sqrt{\kappa t}},$$

where A, B, C and D are constants determined by the experimental conditions.

The data below enable one to calculate them.

(i) $at^{-\frac{1}{2}} = p =$ the penetration factor of von Fürth and Bubanović.

(ii) $A + B\phi\,\frac{p}{2\sqrt{\lambda}} = 0.$

(iii) $C + D\phi\,\frac{p}{2\sqrt{\kappa}} = 0.$

$\phi_0 = 0, \quad \phi_\infty = 1.$

(iv) $A + B = v_0.$

(v) $C \qquad = u_0.$

(vi) $D = \dfrac{-u_0}{\phi\,\frac{p}{2\sqrt{\kappa}}}.$

(vii) $B = \dfrac{v_0}{1 - \phi\,\frac{p}{2\sqrt{\lambda}}}.$

From formula (III), by differentiating the linear functions, we find

$$-\kappa\left\{\frac{2D}{\sqrt{\pi}}e^{-\frac{x^2}{4\kappa t}}\right\}\frac{1}{2\sqrt{\kappa t}} = \lambda\left\{\frac{2B}{\sqrt{\pi}}e^{-\frac{x^2}{4\lambda t}}\right\}\frac{1}{2\sqrt{\lambda t}}$$

at $x = a$,

$$-\kappa^{\frac{1}{2}} De^{-\frac{p^2}{4\kappa}} = \lambda^{\frac{1}{2}} Be^{-\frac{p^2}{4\lambda}}.$$

Substituting for D and B, we arrive at our final formula:

$$\frac{\kappa^{\frac{1}{2}} u_0 e^{-\frac{p^2}{4\kappa}}}{\frac{2}{\sqrt{\pi}}\int_0^{\frac{p}{2\sqrt{\kappa}}} e^{-\beta^2}d\beta} = \frac{\lambda^{\frac{1}{2}} v_0 e^{-\frac{p^2}{4\lambda}}}{1 - \frac{2}{\sqrt{\pi}}\int_0^{\frac{p}{2\sqrt{\lambda}}} e^{-\beta^2}d\beta} \quad \text{.....(IV)}$$

κ can be calculated from this equation if u_0, v_0, λ and p are known. When $\kappa = \lambda$ it simplifies to the old "negative salt" formula

$$\frac{v_0}{v_0 + u_0} = 1 - \frac{2}{\sqrt{\pi}}\int_0^{\frac{x}{2\sqrt{\kappa t}}} e^{-\beta}\,d\beta.$$

VII. Modification of formula (IV).

In its present form, the new formula is not suitable for computations. The arithmetic with formula (IV) takes 50 times as long as that required for formula (II). If this work had to be gone through for each experiment, the beauty of the indicator method would be gone. Much less formidable would be the difficulties of bad electrodes, and of gravimetric analysis, when compared with the labour in the calculations. For example, the right-hand side of the equation is objectionable when the concentration of silver nitrate is small, because then "p" is large, consequently with five figure tables of the probability integral, the error in calculating the denominator may easily exceed 10 %. On the left-hand side, although this difficulty is avoided, we have a worse trouble, since κ, the unknown, can only be arrived at by the method of trial and error.

In the light of these considerations, the calculation of (IV) appeared to be a fruitless effort, but when the formula was placed before Mr Berry, Fellow of King's College, Cambridge, it was a source of much pleasure to a naturalist to see both of these difficulties immediately cleared away, the former by the use of a series formula, the latter by a rearrangement. Both sides of the equation could be so modified that the whole series of calculations could be carried out once and for all.

The modification necessary is given below.

Let $\frac{p}{2\sqrt{\lambda}} = y,$

$\frac{p}{2\sqrt{\kappa}} = z,$

$\phi_q = \frac{2}{\sqrt{\pi}} \int_0^q e^{-\beta^2}\, d\beta,$

then
$$\frac{\phi z}{\frac{p}{2z} u_0 e^{-z^2}} = \frac{1 - \phi y}{\frac{p}{2y} v_0 e^{-y^2}};$$

$$\therefore \frac{v_0}{u_0} = \frac{ye^{y^2} \cdot (1 - \phi y)}{ze^{z^2} \phi z}.$$

The problem of calculating κ is thus reduced to tabulation of these two functions of y and z, and when this has been done, the diffusion coefficient can be worked out with no trouble.

The logarithms of the functions are tabulated, to facilitate arithmetical work

$$f(y) = f\,\frac{p}{2\sqrt{\lambda}} = \log_{10} y + \cdot 4343y^2 + \log_{10}(1 - \phi y).$$

$$f(z) = f\,\frac{p}{2\sqrt{\kappa}} = \log_{10} z + \cdot 4343z^2 + \log_{10}(\phi z).$$

These functions are referred to elsewhere as the λ function and the κ function, because the physical significance of these symbols is an aid in distinguishing them—an association which is lost when y and z are used.

The computation of tables with the κ function is not a difficult matter, but with higher values of y, the probability integral tables are unsuitable for calculating the values of the λ function

$$= \log [ye^{y^2}(1 - \phi y)],$$

replacing $1 - \frac{2}{\sqrt{\pi}} \int_0^y e^{-y^2}\, dy$ by $\frac{2}{\sqrt{\pi}} \int_y^\infty e^{-y^2}\, dy$

we can use the series for $e^{y^2} \int_y^\infty e^{-y^2}\, dy$

$$= \frac{1}{2y} - \frac{1}{2^2y^3} + \frac{1.3}{2^3y^5} - \frac{1.3.5}{2^4y^7} + \ldots.$$

VIII. Experimental verification of new formula.

When calculations are made with these tables the gulf is a wide one between the experimental data and the diffusion coefficient, so the bridge requires testing before we can rely upon it. There are two methods of doing this—the first by trying the effect of different concentrations of silver nitrate, the second

by examining substances which diffuse at a rate differing from that of sodium chloride.

The problem will be treated initially by the negative salt method, on account of the lack of reliable data concerning the diffusion of silver nitrate in gelatin. The figures below, copied from Landolt and Börnstein, refer to water.

Table VI.

Conc. $AgNO_3$	0·02	0·1	0·9	3·9	0·03	0·433
Temperature	12°	12°	12°	12°	7·2°	7·2°
κ (c.g.s.) $10^{-5}\times$	1·2	1·14	1·02	0·62	1·04	0·752

The measurement even for the most dilute solution differs by over 20 % from the coefficient calculated from Nernst's formula [Nernst, p. 273]. It may be remarked when a similar calculation is made for dilute NaCl, the difference is very much smaller—only 2 %.

Table VII.

Diffusion of N NaCl into 1 % agar with different concentrations of $AgNO_3$. d = depths in cm. κ = diffusion coefficient in c.g.s. units $\times 10^5$.

			Concentrations of $AgNO_3$ (normalities)							
Time		Temp.	0·006	0·0154	0·06	0·114	0·006	0·0154	0·06	0·114
hrs.	mins.	°C.	d	d	d	d	κ	κ	κ	κ
16	6	36·4	4·1	3·61	2·78	2·4	1·92	1·91	1·92	1·88
25	3	36·4	5	4·7	3·45	3·02	1·88	2·06	1·90	1·91
39	48	36·4	—	6	4·39	3·8	—	2·13	1·94	1·90

			Concentrations $AgNO_3$					
Time		Temp.	0·006	0·0154	0·1143	0·006	0·0154	0·1143
hrs.	mins.	°C.	d	d	d	κ	κ	κ
15	23	11·5	3	2·7	1·79	1·1	1·11	1·09
24	50	11·7	3·77	3·42	2·24	1·09	1·12	1·07
39	17	10·8	4·75	—	2·85	1·08	—	1·08

			Concentrations $AgNO_3$					
Time		Temp.	0·5	0·1	0·02	0·5	0·1	0·02
hrs.	mins.	°C.	d	d	d	κ	κ	κ
0	54	11·9	0·265	0·475	0·6	1·2	1·2	0·99
2	50	—	0·45	0·765	0·99	1·06	0·99	0·88
6	0	—	0·68	1·16	1·6	1·15	1·09	1·09
8	56	—	0·8	1·35	1·95	1·06	0·99	1·09

The examination of this table points to the conclusion that variations in the concentration of silver nitrate have less effect on "κ" than the errors of an individual observation, when the experiment is made with sodium chloride.

In other words, we have been lucky enough to find an indicator which fulfils the condition postulated, therefore silver nitrate does behave as negative sodium chloride.

We may infer that the diffusion coefficient of silver nitrate probably lies between 1·0 and 1·1 $\times$ 10^{-5} under these experimental conditions.

The study of bodies with diffusion rates differing from this figure will give more interesting results.

Table VIII. *Hydrogen Chloride.*

Diffusion of a 1·03N solution of HCl into 2 % agar with different concentrations of $AgNO_3$. d = depth in cm. κ = coefficient in c.g.s. units × 10^5. Temperature 11·9°.

Time	Concentrations of $AgNO_3$									
hrs. mins.	0·5 d	0·1 d	0·02 d	0·004 d	0·0008 d	0·5 κ	0·1 κ	0·02 κ	0·004 κ	0·0008 κ
0 46	0·22	0·6	0·8	0·1	1·2	0·92	2·24	2·06	2·12	2·20
2 40	0·475	0·96	1·4	1·8	2·15	1·21	1·62	1·81	1·97	2·04
5 59	0·74	1·44	2·15	2·7	3·23	1·31	1·64	1·95	2·02	2·12
8 54	0·9	1·8	2·62	3·31	3·95	1·31	1·73	1·95	2·05	2·14
32 49	1·75	3·5	5·1	—	7·7	1·35	1·76	2·0	—	2·20

This table shows that the negative salt convention breaks down when applied to hydrochloric acid. The variation in κ is far greater than can be accounted for by the experimental error, even though the work was carried on for a brief period of time. The result that $\kappa = 1{\cdot}35 \times 10^{-5}$ (with 0·5N $AgNO_3$) is obviously not a natural constant: it corresponds to an "artefact" in histology, produced by crude technique.

The first step to a more exact analysis of the problem is to work out λ, the diffusion coefficient of silver nitrate. This proved to be quite simple. Since the coefficient of sodium chloride is fairly close to that of silver nitrate, the "negative" method will give reasonable results if the sodium chloride is used as indicator in small concentrations. The results are given below.

Table IX.

Diffusion of 0·4N $AgNO_3$ into a 1 % agar gel, with 0·01N NaCl. d = depth in cm. κ = coefficient × 10^5. Temperature 10°.

Time	Experimental number							
hrs. mins.	1 d	2 d	3 d	4 d	1 κ	2 κ	3 κ	4 κ
16 47	2·4	2·45	2·5	2·5	0·935	0·98	1·02	1·02
24 10	2·97	2·98	3·09	3·0	0·995	1·01	1·08	1·02
40 57	3·82	3·89	3·9	—	0·975	1·01	1·01	—

The diffusion of silver nitrate deserves a more extended study, but these figures seem to be consistent enough for us to use the figure $1{\cdot}01 \times 10^{-5}$ in further calculations—at any rate for a preliminary survey, which is all that can be attempted in this paper.

The figure is probably a fair enough approximation to illustrate distinctly the differences when calculations are made by the new and old formulae.

Table X. *Hydrogen Chloride.*

Diffusion of a 1·035N solution of HCl into agar gels with different concentrations of $AgNO_3$. t = time (hours and minutes). d = depth in cm.

Temp.	Concentrations of $AgNO_3$									
	0·5N		0·1N		0·02N		0·004N		0·0008N	
°C.	t	d	t	d	t	d	t	d	t	d
10·0	16 20	1·29	16 15	2·4	16 7	3·46	16 0	4·3	15 54	5·1
10·3	23 50	1·55	23 45	2·93	24 41	4·2	23 39	5·3	23 37	6·19
9·7	40 29	2·05	40 21	3·83	40 14	5·49	40 7	6·9	40 3	8·05
10·0	48 41	2·265	48 44	4·21	47 48	6·00	48 47	7·6	—	—

The data in this table are probably more reliable than the figures in Table VIII: especially in cases where the depth of penetration was small--though this advantage is offset to some extent by temperature variations. The materials are now complete for carrying out calculations with the aid of our tables of the λ and κ functions.

Table XI.

Illustration of calculations made with the aid of tables of the κ and λ functions where κ is the diffusion coefficient of HCl and $\lambda = 1{\cdot}01 \times 10^{-5}$ that of $AgNO_3$.

Concentration of $AgNO_3$	$0{\cdot}5N$	$0{\cdot}1N$	$0{\cdot}02N$	$0{\cdot}004N$	$0{\cdot}0008N$
"p^2" $= \frac{x^2}{t}$ in seconds	$2{\cdot}86 \times 10^{-5}$	$1{\cdot}0 \times 10^{-4}$	$2{\cdot}05 \times 10^{-4}$	$3{\cdot}3 \times 10^{-4}$	$4{\cdot}51 \times 10^{-4}$
$\log_{10} v_0$	$\bar{1}{\cdot}699$	$\bar{1}{\cdot}0$	$\bar{2}{\cdot}301$	$\bar{3}{\cdot}602$	$\bar{4}{\cdot}903$
$\log_{10} u_0$	0·014	0·014	0·014	0·014	0·014
λ function	$\bar{1}{\cdot}602$	$\bar{1}{\cdot}687$	$\bar{1}{\cdot}716$	$\bar{1}{\cdot}7265$	$\bar{1}{\cdot}733$
κ function	$\bar{1}{\cdot}917$	0·701	1·429	2·1384	2·844
κ by new formula ...	1·38	1·65	1·84	1·96	1·98
κ by old formula ...	1·48	1·72	1·87	1·97+	1·99
Error in old formula	7·2 %	4 %	1·6 %	0·7 %	0·5 %

The same method is applied to magnesium chloride below. The scale of the experiments is too small to eliminate chance errors, but in spite of this the essential difference between the two methods of calculation is well brought out.

Table XII.

Diffusion of N magnesium chloride into 2 % agar with various concentrations of $AgNO_3$. Temperature 11·9°. Time, 5 hours 55 minutes. $\lambda = 1{\cdot}09$ ($\times 10^{-5}$).

Concentration of $AgNO_3$	0·5	0·1	0·02	0·0008
Depth of penetration ...	0·5	1·0	1·4	2·05
κ by new formula ...	0·765	0·835	0·857	0·88
κ by old formula ...	0·628	0·82	0·845	0·875
Error in old formula ...	11 %	1·8 %	1·5 %	0·6 %

With the data given in this table we may bring to a close our compilation of experimental material, and proceed to discuss the results.

IX. Review of the experimental data.

Sodium chloride. The results with this salt are satisfactory when the negative salt convention is used, on account of the lucky coincidence that silver nitrate diffuses as quickly as sodium chloride.

The numerical values of κ at 12°—in the two sets of experiments of Table VII—are $1{\cdot}04$ and $1{\cdot}09 \times 10^{-5}$. Öholm's figure at that temperature, obtained by interpolation, works out at $1{\cdot}06 \times 10^{-5}$ for a normal solution. No stress can be laid on the accuracy of the agreement—the number of experiments is far too small, but it supports Voigtländer's observations on the smallness of the effect of agar gels in retarding diffusion. A 5 % gelatin appears to have a much more noticeable effect.

Hydrogen chloride. The results obtained with this substance are interesting, but the interpretation is doubtful. Table XI shows that the negative salt convention is not reliable, especially when the concentration of silver nitrate is high. Put into words, the table shows that the slower rate of diffusion of silver nitrate helps the hydrogen chloride to advance, and the assumption that the silver nitrate streams up with equal rapidity to meet it cannot give a reliable result. The coefficient appears to be larger than is actually the case, because the weakness of the opposition is ignored.

The difficulty is overcome by the new formula, but it only serves to emphasise the second trouble—the coefficient is far too small when there is a high concentration of silver nitrate in the gel. It is necessary to exercise caution in working out the explanation of this difficulty because we have no right to assume that the silver chloride precipitate has no influence on the rate of diffusion—the precipitate from a strong solution of silver nitrate in the presence of acid may quite easily differ from that formed in a neutral medium. In the absence of direct evidence, this danger can only be mentioned as a possibility—it is not likely that it will prove to be the real explanation. The root of the difficulty is that hydrogen chloride cannot be considered as a simple body. If the compound HCl . $AgNO_3$ was formed, the formula would give accurate results at all concentrations. In reality, the hydrogen ions are not eliminated—therefore the diffusion of fresh hydrogen chloride is retarded. In other words, the "effective concentration" of acid is reduced. Below are given the strengths of HCl which, but for this complication, would give the observed penetration.

Normality of $AgNO_3$	0·5	0·1	0·02	0·004	0·0008
Strength of HCl ...	0·63	0·71	0·82	0·98	1·035

As a matter of fact, this difficulty is rather academic, because the silver nitrate is put in as an indicator—in small concentration—therefore the complications which turn up when its concentration is high are of no practical importance. Nevertheless, it demands our consideration, when deciding what concentration of silver nitrate is the highest we can use. The strength is best kept above 0·0008*N* to ensure the distinctness of the demarcation surface.

Magnesium chloride. The experiments on this substance are on too small a scale to justify any confidence in the absolute numerical values—but as chance errors will affect both results equally, they serve to illustrate the case where the diffusing substance travels more slowly than the silver nitrate—in complete contrast to hydrochloric acid.

In Table XII it will be observed that the negative convention makes the diffusion coefficient of magnesium chloride appear too small, because the "opposition" of the silver is thereby arbitrarily reduced, but the material is not sufficient for further developments of the theory.

Conclusion.

The number of definitions required foils any attempt to give a brief account of certain sections, but rather than repeat lengthy explanations, an illustration is given to show the simplicity of the calculation required in practice.

The indicator technique differs from the standard methods in securing rapidity of execution at the expense of theoretical simplicity. This sacrifice has been of sufficient importance to cause the neglect of the indicator method in the past. The diffusion of two salts resulting in the formation of a zone of precipitate is a problem too full of complications to admit of a rigorous treatment. This disadvantage need not concern us practically, for such a treatment would lead to long calculations consuming more time than that gained by the rapidity of the experimental work. Consequently it is necessary to make some approximations. These are described in sections III and VI, but the following terms are re-defined: "u" = conc. NaCl, "u_0" = conc. NaCl in bath, "κ" = diff. coeff. of NaCl, "v_0" = conc. $AgNO_3$ in jelly, "λ" = diff. coeff. of $AgNO_3$, p = penetration "x" divided by square root of time "t."

The first approximation, called the negative salt convention, leads to the formula

$$\frac{v_0}{u_0+v_0} = u = 1 - \frac{2}{\sqrt{\pi}} \int_0^{\frac{x}{2\sqrt{\kappa t}}} e^{-\beta^2} d\beta. \quad \text{.....(II)}$$

The second approximation, which allows for the different velocity of silver nitrate, leads to the formula

$$\frac{\kappa^{\frac{1}{2}} u_0 e^{-\frac{p^2}{4\kappa}}}{\frac{2}{\sqrt{\pi}} \int_0^{\frac{p}{2\sqrt{\kappa}}} e^{-\beta^2} d\beta} = \frac{\lambda^{\frac{1}{2}} v_0 e^{-\frac{p^2}{4\lambda}}}{1 - \frac{2}{\sqrt{\pi}} \int_0^{\frac{p}{2\sqrt{\lambda}}} e^{-\beta^2} d\beta}. \quad \text{.....(IV)}$$

The calculation of κ with these formulae is a matter of two or three minutes' work at most, but this can be best shown by an illustration, that having once looked up the factor in the table, κ can be worked out in 45 seconds, using formula (II). Given a jelly with $0{\cdot}005N$ silver nitrate, then κ in c.g.s. units is equal to the square of the penetration in cm. divided by the product of the time in hours and 10,000 times the factor—given below the corresponding strength of the salt solution.

Concentration	$5N$	N	$0{\cdot}5N$	$0{\cdot}2N$	$0{\cdot}1N$
Factor	7·8	5·68	4·78	3·65	2·83

The negative salt convention introduces a serious error if the velocity of the diffusing salt is very different from that of silver nitrate unless the concentration of the latter is very small. The method of working out κ, illustrated in Table XI though it takes a few minutes longer, still has the advantage of checking the first result even though the indicator has been diluted enough to conceal the error.

To work out a formula by neglecting secondary phenomena is one thing, but the proof that these can be overlooked is another. The judgment of the

experimental test is a delicate matter for the experimenter, but it is advisable to draw attention to one table, No. XI. The errors in the indicator method are obviously due to the complications caused by silver nitrate and these increase as the strength of the latter is increased; 100 times the normal amount of nitrate gives a difference of 35 %, four times gives 7 %, but when we get down to a 0·005*N* silver nitrate, further dilution is practically without effect.

This shows that we can deliberately increase the magnitude of the "errors of method" but to do this unusual concentrations of silver nitrate are required.

A balanced account of the indicator method would require a description of the advantages comparable in length with the discussion of the difficulties, but the space taken by these leaves little room for the other side of the question. But because there are other diffusion methods, it seems necessary to give some reasons for departing from them.

(1) The measurement of length is direct, it can be made with great accuracy, and the same tube can be measured again at different times. The presence of gelatin or agar-agar, so useful to avoid mechanical mixing, apart from its intrinsic interest, complicates the technique of the standard methods.

(2) The ease with which a large number of experiments can be performed makes the method particularly suitable for an introductory survey even if it gives only a two figure accuracy—and the rough preliminary experiments described above could be greatly improved upon. Specially interesting results could be verified by other methods—but much time would be saved in finding where accurate measurements were most needed.

(3) The fact that no special apparatus is needed enables a worker to determine several diffusion coefficients at a few hours notice, a useful point when these figures are required for another research—for example the comparison with simple diffusion of the rate of absorption of nutrient salts by living tissues.

For educational purposes there is a further advantage due to the simplicity of the apparatus, combined with the fact that the readings can be taken whenever it is convenient.

Data can be obtained to illustrate the methods of handling numerical results in a branch of science which generally demands long experiments before a table or graph can be constructed. The temptation to continue the list of applications must be suppressed, for after all, the special qualities of the method are matters of laboratory technique in which experience is superior to description, but I cannot conclude this account without acknowledging my indebtedness to Professor Stiles, but for whom the work would never have been attempted, and Mr Berry, Fellow of King's College, Cambridge, for his help in dealing with formula (IV).

Appendix.

Table of values of the "λ" function.

$$f\frac{p}{2\sqrt{\lambda}} = \log_{10}\frac{p}{2\sqrt{\lambda}} \cdot e^{\frac{p^2}{4\lambda}}\left(1 - \frac{2}{\sqrt{\pi}}\int_0^{\frac{p}{2\sqrt{\lambda}}} e^{-\beta^2}\,d\beta\right).$$

For values of $\frac{p}{2\sqrt{\lambda}}$

$\frac{p}{2\sqrt{\lambda}}$	$f\frac{p}{2\sqrt{\lambda}}$	$\frac{p}{2\sqrt{\lambda}}$	$f\frac{p}{2\sqrt{\lambda}}$	$\frac{p}{2\sqrt{\lambda}}$	$f\frac{p}{2\sqrt{\lambda}}$
0·4	$\bar{1}$·429	1·8	$\bar{1}$·699	3·4	$\bar{1}$·734
0·5	$\bar{1}$·488	1·9	$\bar{1}$·703	3·6	$\bar{1}$·736
0·6	$\bar{1}$·532	2·0	$\bar{1}$·709	3·8	$\bar{1}$·737
0·7	$\bar{1}$·566	2·1	$\bar{1}$·712	4·0	$\bar{1}$·739
0·8	$\bar{1}$·593	2·2	$\bar{1}$·715	4·5	$\bar{1}$·741
0·9	$\bar{1}$·614	2·3	$\bar{1}$·718	5·0	$\bar{1}$·743
1·0	$\bar{1}$·631	2·4	$\bar{1}$·720	5·5	$\bar{1}$·744
1·1	$\bar{1}$·646	2·5	$\bar{1}$·722	6·0	$\bar{1}$·745
1·2	$\bar{1}$·657	2·6	$\bar{1}$·723	6·5	$\bar{1}$·746
1·3	$\bar{1}$·668	2·7	$\bar{1}$·725	7·0	$\bar{1}$·747
1·4	$\bar{1}$·676	2·8	$\bar{1}$·726	8·0	$\bar{1}$·748
1·5	$\bar{1}$·683	2·9	$\bar{1}$·727	9·0	$\bar{1}$·749
1·6	$\bar{1}$·689	3·0	$\bar{1}$·729	10·0	$\bar{1}$·750
1·7	$\bar{1}$·695	3·2	$\bar{1}$·732	∞	$\bar{1}$·751

Table of values of the "κ" function.

$$f\frac{p}{2\sqrt{\kappa}} = \log_{10}\frac{p}{2\sqrt{\kappa}} \cdot e^{\frac{p^2}{4\kappa}}\left(\frac{2}{\sqrt{\pi}}\int_0^{\frac{p}{2\sqrt{\kappa}}} e^{-\beta^2}\,d\beta\right).$$

For values of $\frac{p}{2\sqrt{\kappa}}$

$\frac{p}{2\sqrt{\kappa}}$	$f\frac{p}{2\sqrt{\kappa}}$	$\frac{p}{2\sqrt{\kappa}}$	$f\frac{p}{2\sqrt{\kappa}}$	$\frac{p}{2\sqrt{\kappa}}$	$f\frac{p}{2\sqrt{\kappa}}$
0·4	$\bar{1}$·3034	1·4	0·956	2·4	2·88
0·5	$\bar{1}$·5240	1·5	1·138	2·5	3·112
0·6	$\bar{1}$·7154	1·6	1·305	2·6	3·36
0·7	$\bar{1}$·8890	1·7	1·48	2·7	3·60
0·8	0·0516	1·8	1·66	2·8	3·85
0·9	0·2074	1·9	1·845	2·9	4·115
1·0	0·3600	2·0	2·036	3·0	4·38
1·1	0·5115	2·1	2·238	3·5	5·864
1·2	0·6637	2·2	2·442	4·0	7·551
1·3	0·8182	2·3	2·659		

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